PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/18, 15/79, 5/06, C07K 14/475, 16/18, C12P 21/02, A61K 38/18, G01N 33/53 A3

(11) International Publication Number: **WO 98/24811**

(43) International Publication Date: 11 June 1998 (11.06.98)

(21) International Application Number: PCT/US97/20888

(22) International Filing Date: 20 November 1997 (20.11.97)

(30) Priority Data:

08/759,657	6 December 1996 (06.12.96)	US
08/933,455	18 September 1997 (18.09.97)	US

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published

With international search report

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(88) Date of publication of the international search report:
1 October 1998 (01.10.98)

(71) Applicant: ZYMOGENETICS, INC. [US/US]; 1201 Eastlake Avenue East, Seattle, WA 98102 (US).

(72) Inventors: GILBERT, Teresa; 246 12th Street S.E., Seattle, WA 98002 (US). CONKLIN, Darrell, C.; Apartment 2, 2332 Minor Avenue East, Seattle, WA 98102 (US). HART, Charles, E.; 20114 183rd Place N.E., Woodinville, WA 98072 (US). NYGAARD, Sean; 13625 S.E. 44th Street, Bellevue, WA 98006 (US). SHEPPARD, Paul, O.; 20717 N.E. 2nd Street, Redmond, WA 98053 (US).

(74) Agent: PARKER, Gary, E.; ZymoGenetics, Inc., 1201 Eastlake Avenue East, Seattle, WA 98102 (US).

(54) Title: VASCULAR ENDOTHELIAL GROWTH FACTOR

(57) Abstract

Growth factors, their component polypeptides, methods of making them, polynucleotides encoding them, and methods of using them are disclosed. The growth factors are homodimeric or heterodimeric proteins having component polypeptide chains that each comprises a sequence of amino acid residues that is at least 80 % identical in amino acid sequence to residues 109 to 197 of SEQ ID NO:2. The growth factors are mitogenic for fibroblasts and smooth muscle cells, and may be used therapeutically or *in vitro* to stimulate cell growth, or to develop inhibitors of cell growth.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

Description

VASCULAR ENDOTHELIAL GROWTH FACTOR

BACKGROUND OF THE INVENTION

In multicellular animals, cell growth, differentiation, and migration are controlled by polypeptide growth factors. These growth factors play a role in both normal development and pathogenesis, including the development of solid tumors.

Polypeptide growth factors influence cellular events by binding to cell-surface receptors, many of which are tyrosine kinases. Binding initiates a chain of signalling events within the cell, which ultimately results in phenotypic changes, such as cell division, protease production, and cell migration.

Growth factors can be classified into families on the basis of structural similarities. One such family, the PDGF (platelet derived growth factor) family, is characterized by a dimeric structure stabilized by disulfide bonds. This family includes PDGF, placental growth factor (PGF), and the vascular endothelial growth factors (VEGFs). Three vascular endothelial growth factors have been identified: VEGF, also known as vascular permeability factor (Dvorak et al., *Am. J. Pathol.* 146:1029-1039, 1995); VEGF-B (Olofsson et al., *Proc. Natl. Acad. Sci. USA* 93:2567-2581, 1996; Hayward et al., WIPO Publication WO 96/27007); and VEGF-C (Joukov et al., *EMBO J.* 15:290-298, 1996). Four VEGF polypeptides (121, 165, 189 and 206 amino acids) arise from alternative splicing of the VEGF mRNA.

VEGFs stimulate the development of vasculature through a process known as angiogenesis, wherein vascular endothelial cells re-enter the cell cycle, degrade underlying basement membrane, and migrate to form new

capillary sprouts. These cells then differentiate, and mature vessels are formed. This process of growth and differentiation is regulated by a balance pro-angiogenic and anti-angiogenic factors. Angiogenesis is central to normal formation and repair of tissue, occuring in embryo development and wound healing. Angiogenesis is also a factor in the development of certain diseases, including solid tumors, rheumatoid arthritis, diabetic retinopathy, macular degeneration, and atherosclerosis.

The role of growth factors in controlling cellular processes makes them likely candidates and targets for therapeutic intervention. Platelet-derived growth factor, for example, has been disclosed for the treatment of periodontal disease (U.S. Patent No. 5,124,316) and gastrointestinal ulcers (U.S. Patent No. 5,234,908). Inhibition of PDGF receptor activity has been shown to reduce intimal hyperplasia in injured baboon arteries (Giese et al., Restenosis Summit VIII, Poster Session #23, 1996). Vascular endothelial growth factors (VEGFs) have been shown to promote the growth of blood vessels in ischemic limbs (Isner et al., *The Lancet* 348:370-374, 1996), and have been proposed for use as wound-healing agents, for treatment of periodontal disease, for promoting endothelialization in vascular graft surgery, and for promoting collateral circulation following myocardial infarction (WIPO Publication No. WO 95/24473; U.S. Patent No. 5,219,739). VEGFs are also useful for promoting the growth of vascular endothelial cells in culture. A soluble VEGF receptor (soluble flt-1) has been found to block binding of VEGF to cell-surface receptors and to inhibit the growth of vascular tissue in vitro (*Biotechnology News* 16(17):5-6, 1996).

SUMMARY OF THE INVENTION

Within one aspect of the present invention there are provided isolated polypeptides comprising a sequence of amino acid residues that is at least 80% identical in amino acid sequence to residues 109 to 197 of SEQ ID NO:2, wherein the polypeptides dimerize to form homodimeric or heterodimeric proteins that are mitogenic for fibroblasts or smooth muscle cells. Within one embodiment of the invention, the polypeptides are at least 90% identical in amino acid sequence to residues 109 to 197 of SEQ ID NO:2. Within another embodiment, the polypeptides further comprise a Balbiani ring motif carboxyl-terminal to the sequence of amino acid residues. Within additional embodiments the polypeptides comprise a sequence of amino acid residues as shown in SEQ ID NO:2 selected from the group consisting of residues 109-205, residues 85-205, residues 22-205, residues 1-205, residues 109-354, residues 85-354, residues 22-354, and residues 1-354. The polypeptides may further comprise an affinity tag such as, for example, polyhistidine, protein A, glutathione S transferase, substance P, or an immunoglobulin heavy chain constant region.

Within a second aspect of the invention there are provided isolated protein dimers having two polypeptide chains as disclosed above, wherein the proteins are mitogenic for fibroblasts or smooth muscle cells. The proteins include heterodimers and homodimers of the polypeptides disclosed above.

Within a third aspect of the invention there are provided polypeptides produced by a method comprising the steps of: (a) culturing a cell containing a DNA construct comprising the following operably linked elements: a transcription promoter; a DNA segment encoding a polypeptide that is at least 80% identical to the amino acid sequence of SEQ ID NO:2 from residue 22 to residue

354; and a transcription terminator; and (b) isolating the polypeptide encoded by the DNA segment and produced by the cell. Within one embodiment, the DNA construct further comprises a secretory signal sequence operably linked to the DNA segment. Within another embodiment, the DNA segment encodes a polypeptide that is at least 90% identical in amino acid sequence to residues 22 to 354 of SEQ ID NO:2. Within a further embodiment, the DNA segment encodes a polypeptide that is at least 95% identical in amino acid sequence to residues 22 to 354 of SEQ ID NO:2.

Within a fourth aspect, the invention provides dimeric proteins produced by a method comprising the steps of (a) culturing a cell containing a DNA construct comprising the following operably linked elements: a transcription promoter; a secretory signal sequence; a DNA segment encoding a polypeptide that is at least 80% identical to the amino acid sequence of SEQ ID NO:2 from residue 22 to residue 354; and a transcription terminator, whereby the DNA segment is expressed and the polypeptide is dimerized to form a dimeric protein, and (b) isolating the dimeric protein from the cell. Within one embodiment, the DNA segment encodes a polypeptide that is at least 90% identical in amino acid sequence to residues 22 to 354 of SEQ ID NO:2. Within another embodiment, the DNA segment encodes a polypeptide that is at least 95% identical in amino acid sequence to residues 22 to 354 of SEQ ID NO:2.

Within a fifth aspect, the invention provides an isolated polynucleotide encoding a polypeptide as disclosed above. Within one embodiment, the polynucleotide is DNA. Within another embodiment, the polynucleotide is from 660 base pairs to 1062 base pairs in length.

Within a sixth aspect of the invention there are provided expression vectors which comprise the following operably linked elements: a transcription promoter; a DNA segment encoding a polypeptide as disclosed above; and a

transcription terminator. The expression vectors may further comprise a secretory signal sequence operably linked to the DNA segment.

Within a seventh aspect of the invention there is provided a cultured cell into which has been introduced an expression vector as disclosed above, wherein said cell expresses the DNA segment and produces a polypeptide encoded by the DNA segment. Within one embodiment, the expression vector comprises a secretory signal sequence operably linked to the DNA segment, and the cell expresses the DNA segment and secretes a polypeptide encoded by the DNA segment in the form of a dimeric protein.

Within additional aspects of the invention there are provided antibodies that specifically bind to the polypeptides and protein dimers disclosed above.

A further aspect of the invention provides a method of promoting cell growth, comprising incubating eukaryotic cells in a culture medium comprising a dimeric protein as disclosed above in an amount sufficient to stimulate mitogenesis in said cells. Within one embodiment, the cells are fibroblasts or smooth muscle cells.

An additional aspect of the present invention provides methods for identifying antagonists of the dimeric proteins disclosed above. Within one embodiment, there is provided a method of identifying an inhibitor of cell mitogenesis, comprising providing cells responsive to a dimeric protein as disclosed above, culturing a first portion of the cells in the presence of the dimeric protein, culturing a second portion of the cells in the presence of the dimeric protein and a test sample, and detecting a decrease in a cellular response of the second portion of the cells as compared to the first portion of the cells. Within a second embodiment, there is provided a method of detecting a growth factor antagonist, comprising assaying a test sample for the ability to

reduce binding of a dimeric protein as disclosed above to a receptor.

These and other aspects of the invention will become evident upon reference to the following detailed description of the invention and the attached drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a Hopp/Woods hydrophilicity profile of the zvegf2 protein sequence shown in SEQ ID NO:2. The profile is based on a sliding six-residue window. Buried G, S, and T residues and exposed H, Y, and W residues were ignored. These residues are indicated in the figure by lower case letters.

Fig. 2 illustrates a Western blot of recombinant zvegf2. Lane 1, conditioned media from control transfected cells. Lane 2, zvegf2-T conditioned media. Lane 3, zvegf2-FL conditioned media. Lane 4, his-tagged MPL receptor, 1000 ng. Lane 5, his-tagged MPL receptor, 100 ng. Lane 6, his-tagged MPL receptor, 10 ng.

DETAILED DESCRIPTION OF THE INVENTION

Prior to setting forth the invention in detail, it may be helpful to the understanding thereof to define the following terms:

The term "affinity tag" is used herein to denote a peptide segment that can be attached to a polypeptide to provide for purification of the polypeptide or provide sites for attachment of the polypeptide to a substrate. In principal, any peptide or protein for which an antibody or other specific binding agent is available can be used as an affinity tag. Affinity tags include a poly histidine tract, protein A (Nilsson et al., *EMBO J.* 4:1075 1985; Nilsson et al., *Methods Enzymol.* 198:3, 1991), glutathione S transferase (Smith and Johnson, *Gene* 67:31, 1988), substance P, Flag™ peptide (Hopp et al., *Biotechnology* 6:1204-1210, 1988; available from Eastman

Eastman Kodak Co., New Haven, CT), streptavidin binding peptide, or other antigenic epitope or binding domain. See, in general Ford et al., *Protein Expression and Purification* 2: 95-107, 1991, which is incorporated herein by reference. DNAs encoding affinity tags are available from commercial suppliers (e.g., Pharmacia Biotech, Piscataway, NJ).

The term "allelic variant" is used herein to denote any of two or more alternative forms of a gene occupying the same chromosomal locus. Allelic variation arises naturally through mutation, and may result in phenotypic polymorphism within populations. Gene mutations can be silent (no change in the encoded polypeptide) or may encode polypeptides having altered amino acid sequence. The term allelic variant is also used herein to denote a protein encoded by an allelic variant of a gene.

The terms "amino-terminal" and "carboxyl-terminal" are used herein to denote positions within polypeptides and proteins. Where the context allows, these terms are used with reference to a particular sequence or portion of a polypeptide or protein to denote proximity or relative position. For example, a certain sequence positioned carboxyl-terminal to a reference sequence within a protein is located proximal to the carboxyl terminus of the reference sequence, but is not necessarily at the carboxyl terminus of the complete protein.

The term "expression vector" is used to denote a DNA molecule, linear or circular, that comprises a segment encoding a polypeptide of interest operably linked to additional segments that provide for its transcription. Such additional segments include promoter and terminator sequences, and may also include one or more origins of replication, one or more selectable markers, an enhancer, a polyadenylation signal, etc. Expression vectors are

generally derived from plasmid or viral DNA, or may contain elements of both.

The term "isolated", when applied to a polynucleotide, denotes that the polynucleotide has been removed from its natural genetic milieu and is thus free of other extraneous or unwanted coding sequences, and is in a form suitable for use within genetically engineered protein production systems. Such isolated molecules are those that are separated from their natural environment and include cDNA and genomic clones. Isolated DNA molecules of the present invention are free of other genes with which they are ordinarily associated, but may include naturally occurring 5' and 3' untranslated regions such as promoters and terminators. The identification of associated regions will be evident to one of ordinary skill in the art (see for example, Dynan and Tijan, *Nature* 316:774-78, 1985).

An "isolated" polypeptide or protein is a polypeptide or protein that is found in a condition other than its native environment, such as apart from blood and animal tissue. In a preferred form, the isolated polypeptide is substantially free of other polypeptides, particularly other polypeptides of animal origin. It is preferred to provide the polypeptides in a highly purified form, i.e. greater than 95% pure, more preferably greater than 99% pure. When used in this context, the term "isolated" does not exclude the presence of the same polypeptide in alternative physical forms, such as dimers or alternatively glycosylated or derivatized forms.

"Operably linked", when referring to DNA segments, indicates that the segments are arranged so that they function in concert for their intended purposes, e.g., transcription initiates in the promoter and proceeds through the coding segment to the terminator.

A "polynucleotide" is a single- or double-stranded polymer of deoxyribonucleotide or ribonucleotide

bases read from the 5' to the 3' end. Polynucleotides include RNA and DNA, and may be isolated from natural sources, synthesized in vitro, or prepared from a combination of natural and synthetic molecules.

The term "promoter" is used herein for its art-recognized meaning to denote a portion of a gene containing DNA sequences that provide for the binding of RNA polymerase and initiation of transcription. Promoter sequences are commonly, but not always, found in the 5' non-coding regions of genes.

A "secretory signal sequence" is a DNA sequence that encodes a polypeptide (a "secretory peptide") that, as a component of a larger polypeptide, directs the larger polypeptide through a secretory pathway of a cell in which it is synthesized. The larger polypeptide is commonly cleaved to remove the secretory peptide during transit through the secretory pathway.

The present invention provides novel growth factor polypeptides and proteins. This novel growth factor, termed "zvegf2", exhibits significant amino acid sequence homology to the previously described vascular endothelial growth factors (Dvorak et al.,ibid.; Olofsson et al., ibid.; Joukov et al., ibid.). For example, one of the polypeptides of the present invention is approximately 40% identical to VEGF-C (Joukov et al., ibid.) when the sequences are aligned to produce a 269 amino acid residue overlap. The VEGFs are homodimeric or heterodimeric proteins, the monomer subunits of which include a receptor-binding domain characterized by a paired, twisted beta sheet structure stabilized by conserved cysteine residues. Referring to SEQ ID NO:2, these conserved cysteine residues are at positions 111, 136, 142, 145, 147, 153, 160, and 191. This domain is further characterized by three beta strand connecting loops (approximately residues 119-134, 147-152, and 166-175 of SEQ ID NO:2). Within SEQ ID NO:2, the receptor binding

domain extends from approximately residue 109 (Thr) through residue 197 (Arg). A polypeptide consisting of this sequence of amino acids is referred to herein as zvegf2(109-197). Those skilled in the art will recognize that domain boundaries are approximate, and that one or a few residues may be removed or substituted at either end without destroying biological activity.

Unlike the previously described VEGFs, the polypeptides and proteins of the present invention stimulate the growth of cultured smooth muscle cells and fibroblasts. In contrast, the previously described VEGFs are specific to endothelial cells (reviewed by Engler, Circulation 94:1496-1498, 1996) and certain tumor and hematopoietic cell types. Zvegf2 polypeptides and proteins may also stimulate growth of other cell types, including endothelial and dendritic cells.

Additional structural features of the zvegf2 primary translation product include an amino-terminal secretory peptide extending from residue 1 (Met) through residue 20 (Gln) of SEQ ID NO:2. Potential cleavage sites exist at residues 108-109 (Arg-Thr) and at residues 84-85 (His-Arg), suggesting a possible propeptide or other amino-terminal processing. The carboxyl-terminal region of the primary translation product comprises four cysteine-rich domains. Referring to SEQ ID NO:2, the first extends from residue 206 to about residue 256. The second cysteine-rich domain is a Balbiani ring motif extending from residues 257 through approximately residue 274 of SEQ ID NO:2. This motif is characterized by the consensus Balbiani ring sequence Cys Xaa$_2$-Cys Xaa$_3$-Cys Xaa$_3$-Cys (SEQ ID NO:3). A Balbiani ring like cysteine-rich motif extends from approximately residue 275 to approximately residue 294. A fourth cysteine-rich domain, containing eight Cys residues, extends from approximately residue 295 to the carboxyl terminus of the primary translation product. While not wishing to be bound by

theory it is believed that the primary translation product is naturally processed in eukaryotic cells to remove the signal peptide, and that additional processing may remove the putative propeptide and/or the C-terminal region (including the Balbiani ring motif) during secretion. Balbiani ring sequences are generally believed to provide for one or more of entry into the secretory pathway, processing, assembly, transport and storage of the polypeptide, and, as such, they are useful in the production of certain zvegf2 polypeptides within the present invention. However, the present invention is not limited to the expression of the full-length sequence shown in SEQ ID NO:1. A number of truncated zvegf2 polynucleotides and polypeptides are provided by the present invention. These polypeptides can be produced by expressing polynucleotides encoding them in a variety of host cells. In many cases, the structure of the final polypeptide product will result from processing of the nascent polypeptide chain by the host cell, thus the final sequence of a zvegf2 polypeptide produced by a host cell will not always correspond to the full sequence encoded by the expressed polynucleotide. For example, expressing the full-length sequence shown in SEQ ID NO:1 in a cultured mammalian cell is expected to result in removal of at least the secretory peptide, while the same polypeptide produced in a prokaryotic host would not be expected to be cleaved. By selecting particular combinations of polynucleotide and host cell, a variety of zvegf2 polypeptides can thus be produced. In addition, zvegf2 polypeptides can be produced by other known methods, such as solid phase synthesis, methods for which are well known in the art. Particularly preferred zvegf2 polypeptides are shown below in Table 1. These polypeptides are designated by the positions of their amino- and carboxyl-terminal residues as shown in SEQ ID NO:2. Differential processing of individual chains may result in

heterogeneity of expressed polypeptides and the production of heterodimeric zvegf2 proteins.

Table 1

zvegf2(109-197)
zvegf2(109-205)
zvegf2(109-218)
zvegf2(109-220)
zvegf2(109-274)
zvegf2(109-354)
zvegf2(85-197)
zvegf2(85-205)
zvegf2(85-218)
zvegf2(85-220)
zvegf2(85-274)
zvegf2(85-354)
zvegf2(22-197)
zvegf2(22-205)
zvegf2(22-218)
zvegf2(22-220)
zvegf2(22-274)
zvegf2(22-354)
zvegf2(1-197)
zvegf2(1-205)
zvegf2(1-218)
zvegf2(1-274)
zvegf2(1-354)

Those skilled in the art will recognize that useful polypeptides having amino and/or carboxyl termini intermediate to those of the polypeptides shown in Table 1 can also be prepared. Such intermediate polypeptides are prepared using the methods disclosed above, including direct expression, expression with subsequent proteolysis, and in vitro synthesis.

Dimerization of zvegf2 polypeptides, either in vivo or in vitro, generates biologically active proteins. Dimeric proteins of the present invention include both homodimers and heterodimers of zvegf2 polypeptides disclosed above. Zvegf2 proteins of the present invention are characterized by their ability to stimulate mitogenesis in mesenchymal cells (including fibroblasts and smooth muscle cells). These proteins may also induce vascular permeability in animals. Mitogenic activity can be measured using known assays, including ^{3}H-thymidine incorporation assays (as disclosed by, e.g., Raines and Ross, *Methods Enzymol.* 109:749-773, 1985) or cell counts. A preferred mitogenesis assay measures the incorporation of [^{3}H]-thymidine into vascular smooth muscle cells or fibroblasts. Within a typical such assay, human dermal fibroblasts are plated at a density of approximately 8,000 cells/well in 24-well culture plates and grown for approximately 72 hours in a suitable culture medium, such as DMEM containing 10% fetal calf serum. The cells are allowed to become quiescent, then exposed to a test solution. After a period of time, typically about 24 hours, [^{3}H]-thymidine is added and incubation is continued to allow growing cells to incorporate the label. The cells are then harvested, and incorporation of label is determined according to standard procedures. See also, Gospodarowicz et al., *J. Cell. Biol.* 70:395-405, 1976; Ewton and Florini, *Endocrinol.* 106:577-583, 1980; and Gospodarowicz et al., *Proc. Natl. Acad. Sci. USA* 86:7311-7315, 1989.

Induction of vascular permeability is measured in assays designed to detect leakage of protein from the vasculature of a test animal (e.g., mice or guinea pig) after administration of a test compound (Miles and Miles, *J. Physiol.* 118:228-257, 1952; Feng et al., *J. Exp. Med.* 183:1981-1986, 1996).

The present invention also provides polynucleotide molecules, including DNA and RNA molecules, that encode the zvegf2 polypeptides disclosed above. Those skilled in the art will readily recognize that, in view of the degeneracy of the genetic code, considerable sequence variation is possible among these polynucleotide molecules. SEQ ID NO:14 is a degenerate DNA sequence that encompasses all DNAs that encode the zvegf2 polypeptide of SEQ ID NO: 2. Those skilled in the art will recognize that the degenerate sequence of SEQ ID NO:14 also provides all RNA sequences encoding SEQ ID NO:2 by substituting U for T. Thus, zvegf2 polypeptide-encoding polynucleotides comprising nucleotide 325 to nucleotide 891 of SEQ ID NO: 14 and their RNA equivalents are contemplated by the present invention. Preferred such sequences include nucleotides 325-615, 325-654, 325-660, 325-822, 325-1062, 253-591, 253-615, 253-654, 253-660, 253-822, 253-1062, 64-591, 64-615, 64-654, 64-660, 64-822, 64-1062, 1-615, 1-654, 1-660, 1-822, and 1-1062 of SEQ ID NO:14. Table 2 sets forth the one-letter codes used within SEQ ID NO:14 to denote degenerate nucleotide positions. "Resolutions" are the nucleotides denoted by a code letter. "Complement" indicates the code for the complementary nucleotide(s). For example, the code Y denotes either C or T, and its complement R denotes A or G, A being complementary to T and G being complementary to C.

TABLE 2

Nucleotide	Resolutions	Complement	Resolutions
A	A	T	T
C	C	G	G
G	G	C	C
T	T	A	A
R	A|G	Y	C|T
Y	C|T	R	A|G
M	A|C	K	G|T
K	G|T	M	A|C
S	C|G	S	C|G
W	A|T	W	A|T
H	A|C|T	D	A|G|T
B	C|G|T	V	A|C|G
V	A|C|G	B	C|G|T
D	A|G|T	H	A|C|T
N	A|C|G|T	N	A|C|G|T

The degenerate codons used in SEQ ID NO:14, encompassing all possible codons for a given amino acid, are set forth in Table 3, below.

TABLE 3

Amino Acid	One-Letter Code	Codons	Degenerate Codon
Cys	C	TGC TGT	TGY
Ser	S	AGC AGT TCA TCC TCG TCT	WSN
Thr	T	ACA ACC ACG ACT	CAN
Pro	P	CCA CCC CCG CCT	CCN
Ala	A	GCA GCC GCG GCT	GCN
Gly	G	GGA GGC GGG GGT	GGN
Asn	N	AAC AAT	AAY
Asp	D	GAC GAT	GAY
Glu	E	GAA GAG	GAR
Gln	Q	CAA CAG	CAR
His	H	CAC CAT	CAY
Arg	R	AGA AGG CGA CGC CGG CGT	MGN
Lys	K	AAA AAG	AAR
Met	M	ATG	ATG
Ile	I	ATA ATC ATT	ATH
Leu	L	CTA CTC CTG CTT TTA TTG	YTN
Val	V	GTA GTC GTG GTT	GTN
Phe	F	TTC TTT	TTY
Tyr	Y	TAC TAT	TAY
Trp	W	TGG	TGG
Ter	.	TAA TAG TGA	TRR
Asn or Asp	B		RAY
Glu or Gln	Z		SAR
Any	X		NNN
Gap	-	---	

One of ordinary skill in the art will appreciate that some ambiguity is introduced in determining a degenerate codon, representative of all possible codons encoding each amino acid. For example, the degenerate codon for serine (WSN) can, in some circumstances, encode arginine (AGR), and the degenerate codon for arginine (MGN) can, in some circumstances, encode serine (AGY). A similar relationship exists between codons encoding phenylalanine and leucine. Thus, some polynucleotides encompassed by the degenerate sequence may encode variant amino acid sequences, but one of ordinary skill in the art can easily identify such variant sequences by reference to the amino acid sequence of SEQ ID NO: 2. Variant sequences can be readily tested for functionality as described herein.

Within preferred embodiments of the invention the isolated polynucleotides will hybridize to similar sized regions of SEQ ID NO:1, or a sequence complementary thereto, under stringent conditions. In general, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Typical stringent conditions are those in which the salt concentration is about 0.02 M at pH 7 and the temperature is at least about 60°C.

As previously noted, the isolated polynucleotides of the present invention include DNA and RNA. Methods for preparing DNA and RNA are well known in the art. It is generally preferred to isolate RNA from heart, including whole heart tissue extracts or heart cells (e.g., purified by cytosol), although DNA can also be prepared using RNA from other tissues (including lung, skeletal muscle, uterus, small intestine, and colon) or isolated as genomic DNA. Total RNA can be prepared using

guanidine HCl extraction followed by isolation by centrifugation in a CsCl gradient (Chirgwin et al., *Biochemistry* 18:52-94, 1979). Poly (A)$^+$ RNA is prepared from total RNA using the method of Aviv and Leder (*Proc. Natl. Acad. Sci. USA* 69:1408-1412, 1972). Complementary DNA (cDNA) is prepared from poly(A)$^+$ RNA using known methods. Polynucleotides encoding zvegf2 polypeptides are then identified and isolated by, for example, hybridization or PCR.

Those skilled in the art will recognize that the sequences disclosed in SEQ ID NOS:1, 2, and 14 represent a single allele of human zvegf2. Allelic variants of these sequences can be cloned by probing cDNA or genomic libraries from different individuals according to standard procedures.

The present invention further provides counterpart polypeptides and polynucleotides from other species ("species orthologs"). Of particular interest are zvegf2 polypeptides from other mammalian species, including murine, porcine, ovine, bovine, canine, feline, equine, and other primate polypeptides. Species orthologs of human zvegf2 can be cloned using information and compositions provided by the present invention in combination with conventional cloning techniques. For example, a cDNA can be cloned using mRNA obtained from a tissue or cell type that expresses zvegf2, such as heart, skeletal muscle, uterus, and small intestine. Suitable sources of mRNA can be identified by probing Northern blots with probes designed from the sequences disclosed herein. A library is then prepared from mRNA of a positive tissue or cell line. A zvegf2-encoding cDNA can then be isolated by a variety of methods, such as by probing with a complete or partial human cDNA or with one or more sets of degenerate probes based on the disclosed sequences. A cDNA can also be cloned using the polymerase chain reaction, or PCR (Mullis, U.S. Patent No.

4,681,202 , using primers designed from the representative human zvegf2 sequence disclosed herein. Within an additional method, the cDNA library can be used to transform or transfect host cells, and expression of the cDNA of interest can be detected with an antibody to zvegf2 polypeptide. Similar techniques can also be applied to the isolation of genomic clones.

Those skilled in the art will recognize that there is considerable latitude in amino acid sequence, and that equivalent polypeptides can be produced by engineering amino acid changes into the representative human polypeptide sequence shown in SEQ ID NO:2 or an allelic variant or species ortholog thereof. It is preferred that these engineered variant polypeptides are at least 80% identical within the receptor binding domain corresponding to residues 109-197 of SEQ ID NO:2. Such polypeptides will more preferably be at least 90% identical, and most preferably 95% or more identical to SEQ ID NO:2 within the receptor binding domain. Within certain embodiments of the invention, the polypeptides are at least 80%, more preferably at least 90%, and most preferably at least 95% identical in sequence throughout their length to the corresponding region of SEQ ID NO:2. Percent sequence identity is determined by conventional methods. See, for example, Altschul et al., *Bull. Math. Bio.* 48: 603-616, 1986 and Henikoff and Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915-10919, 1992. Briefly, two amino acid sequences are aligned to optimize the alignment scores using a gap opening penalty of 10, a gap extension penalty of 1, and the "blosum 62" scoring matrix of Henikoff and Henikoff (ibid. as shown in Table 1 amino acids are indicated by the standard one letter codes).

The percent identity is then calculated as:

$$\frac{\text{Total number of identical matches}}{\text{[length of the longer sequence plus the number of gaps introduced into the longer sequence in order to align the two sequences]}} \times 100$$

Table 4

	A	R	N	D	C	Q	E	G	H	I	L	K	M	F	P	S	T	W	Y	V
A	4																			
R	-1	5																		
N	2	0	6																	
D	2	-2	1	6																
C	0	-3	-3	-3	9															
Q	1	1	0	0	-3	5														
E	-1	0	0	2	-4	2	5													
G	0	-2	0	-1	-3	-2	-2	6												
H	-2	0	1	-1	-3	0	0	-2	8											
I	-1	-3	-3	-3	-1	-3	-3	-4	-3	4										
L	-1	-2	-3	-4	-1	-2	-3	-4	-3	2	4									
K	-1	2	0	-1	-3	1	1	-2	-1	-3	-2	5								
M	-1	-1	-2	-3	-1	0	-2	-3	-2	1	2	-1	5							
F	-2	-3	-3	-3	-2	-3	-3	-3	-1	0	0	-3	0	6						
P	-1	-2	-2	-1	-3	-1	-1	-2	-2	-3	-3	-1	-2	-4	7					
S	1	-1	1	0	-1	0	0	0	-1	-2	-2	0	-1	-2	-1	4				
T	0	-1	0	-1	-1	-1	-1	-2	-2	-1	-1	-1	-1	-2	-1	1	5			
W	-3	-3	-4	-4	-2	-2	-3	-2	-2	-3	-2	-3	-1	1	-4	-3	-2	11		
Y	-2	-2	-2	-3	-2	-1	-2	-3	-2	-1	-1	-2	-1	3	-3	-2	-2	2	7	
V	0	-3	-3	-3	-1	-2	-2	-3	-3	3	1	-2	1	-1	-2	-2	0	-3	-1	4

Sequence identity of polynucleotide molecules is determined by similar methods using a ratio as disclosed above.

Engineered variant zvegf2 polypeptides are characterized as having one or more amino acid substitutions, deletions or additions. These changes are preferably of a minor nature, that is conservative amino acid substitutions (see Table 5) and other substitutions that do not significantly affect the folding or activity of the polypeptide; small deletions, typically of one to about 30 amino acids; and small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue, a small linker peptide of up to about 20-25 residues, or an affinity tag. Polypeptides comprising affinity tags can further comprise a proteolytic cleavage site between the zvegf2 polypeptide and the affinity tag. Preferred such sites include thrombin cleavage sites and factor Xa cleavage sites.

Table 5

Conservative amino acid substitutions

Basic:	arginine
	lysine
	histidine
Acidic:	glutamic acid
	aspartic acid
Polar:	glutamine
	asparagine
Hydrophobic:	leucine
	isoleucine
	valine
Aromatic:	phenylalanine
	tryptophan
	tyrosine

Table 5, continued

Small	glycine
	alanine
	serine
	threonine
	methionine

In addition to the 20 standard amino acids, non-standard amino acids (such as 4-hydroxyproline, 6-*N*-methyl lysine, 2-aminoisobutyric acid, isovaline and α-methyl serine) may be substituted for amino acid residues of zvegf2 polypeptides. A limited number of non-conservative amino acids, amino acids that are not encoded by the genetic code, and unnatural amino acids may be substituted for zvegf2 amino acid residues. "Unnatural amino acids" have been modified after protein synthesis, and/or have a chemical structure in their side chain(s) different from that of the standard amino acids. Unnatural amino acids can be chemically synthesized or obtained commercially, and include pipecolic acid, thiazolidine carboxylic acid, dehydroproline, 3- and 4-methylproline, and 3,3-dimethylproline. The inclusion of non-standard amino acid residues may result in increased *in vivo* half-life.

Essential amino acids in the polypeptides of the present invention can be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, *Science* 244, 1081-1085, 1989; Bass et al., *Proc. Natl. Acad. Sci. USA* 88:4498-4502, 1991). In the latter technique, single alanine mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological activity (e.g., ^{3}H-thymidine incorporation into vascular smooth muscle cells or fibroblasts) to identify amino acid

residues that are critical to the activity of the molecule. The identities of essential amino acids can also be inferred from analysis of homologies with vascular endothelial growth factors.

Multiple amino acid substitutions can be made and tested using known methods of mutagenesis and screening, such as those disclosed by Reidhaar-Olson and Sauer (*Science* 241:53-57, 1988) or Bowie and Sauer (*Proc. Natl. Acad. Sci. USA* 86:2152-2156, 1989). Briefly, these authors disclose methods for simultaneously randomizing two or more positions in a polypeptide, selecting for functional polypeptide, and then sequencing the mutagenized polypeptides to determine the spectrum of allowable substitutions at each position. Other methods that can be used include phage display (e.g., Lowman et al., *Biochem.* 30:10832-10837, 1991; Ladner et al., U.S. Patent No. 5,223,409; Huse, WIPO Publication WO 92/06204) and region-directed mutagenesis (Derbyshire et al., *Gene* 46:145, 1986; Ner et al., *DNA* 7:127, 1988).

Amino acid sequence changes are made in zvegf2 polypeptides so as to minimize disruption of higher order structure essential to biological activity. In this regard, it is generally preferred to retain the cysteine residues at positions 67, 111, 117, 136, 142, 145, 146, 153, 189, and 191 of SEQ ID NO:2 and to retain the overall hydrophilicity profile of the natural sequence. A hydrophilicity profile of the sequence shown in SEQ ID NO:2 is shown in Fig. 1.

Within certain embodiments of the invention, the zvegf2 polynucleotides encode primary translation products comprising one or more C-terminal Balbiani rings. As noted above, Balbiani rings are believed to facilitate the intracellular transport and storage of proteins, possibly by maintaining protein solubility (Paulsson et al., *J. Mol. Biol.* 211:331-349, 1990). It may thus be beneficial to include one or more Balbiani

ring sequences within a polynucleotide of the present invention. Such sequences will commonly encode up to 6, more commonly not more than 4, Balbiani rings, although 10 or more such rings can be included. Proteins having as many as 62 Balbiani rings are known (Paulsson et al., J. Mol. Biol. 211:331-349, 1990).

Mutagenesis methods as disclosed above can be combined with high-throughput screening methods to detect biological activity of zvegf2 variant polypeptides. Preferred assays in this regard include mitogenesis assays, which can be run in a 96-well format. Screens designed to measure activation of receptor-linked pathways can also be employed. Such assays typically measure the expression of a reporter gene (encoding, for example, luciferase or green fluorescent protein) that is linked to a serum response element. Mutagenized DNA molecules that encode active zvegf2 polypeptides can be recovered from the host cells and rapidly sequenced using modern equipment. These methods allow the rapid determination of the importance of individual amino acid residues in a polypeptide of interest, and can be applied to polypeptides of unknown structure.

Using the methods discussed above, one of ordinary skill in the art can prepare a variety of polypeptide fragments or variants of SEQ ID NO:2 that retain the mitogenic activity of wild-type zvegf2.

For any vegf2 polypeptide, including variants and fusion proteins, one of ordinary skill in the art can readily generate a fully degenerate polynucleotide sequence encoding that variant using the information set forth in Tables 2 and 3, above.

The zvegf2 polypeptides of the present invention, including full length polypeptides, biologically active fragments, and fusion polypeptides can be produced in genetically engineered host cells according to conventional techniques. Suitable host

cells are those cell types that can be transformed or transfected with exogenous DNA and grown in culture, and include bacteria, fungal cells, and cultured higher eukaryotic cells. Eukaryotic cells, particularly cultured cells of multicellular organisms, are preferred. Techniques for manipulating cloned DNA molecules and introducing exogenous DNA into a variety of host cells are disclosed by Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989, and Ausubel et al., ibid.

In general, a DNA sequence encoding a zvegf2 polypeptide is operably linked to other genetic elements required for its expression, generally including a transcription promoter and terminator, within an expression vector. The vector will also commonly contain one or more selectable markers and one or more origins of replication, although those skilled in the art will recognize that within certain systems selectable markers may be provided on separate vectors, and replication of the exogenous DNA may be provided by integration into the host cell genome. Selection of promoters, terminators, selectable markers, vectors and other elements is a matter of routine design within the level of ordinary skill in the art. Many such elements are described in the literature and are available through commercial suppliers.

To direct a zvegf2 polypeptide into the secretory pathway of a host cell, a secretory signal sequence (also known as a leader sequence, prepro sequence or pre sequence) is provided in the expression vector. The secretory signal sequence may be that of zvegf2, or may be derived from another secreted protein (e.g., t-PA) or synthesized de novo. The secretory signal sequence is operably linked to the zvegf2 DNA sequence, i.e., the two sequences are joined in the

correct reading frame and positioned to direct the newly synthesized polypeptide into the secretory pathway of the host cell. Secretory signal sequences are commonly positioned 5' to the DNA sequence encoding the polypeptide of interest, although certain signal sequences may be positioned elsewhere in the DNA sequence of interest (see, e.g., Welch et al., U.S. Patent No. 5,037,743; Holland et al., U.S. Patent No. 5,143,830).

Cultured mammalian cells are preferred hosts within the present invention. Methods for introducing exogenous DNA into mammalian host cells include calcium phosphate-mediated transfection (Wigler et al., *Cell* 14:725, 1978; Corsaro and Pearson, *Somatic Cell Genetics* 7:603, 1981: Graham and Van der Eb, *Virology* 52:456, 1973), electroporation (Neumann et al., *EMBO J.* 1:841-845, 1982), DEAE-dextran mediated transfection (Ausubel et al., eds., *Current Protocols in Molecular Biology*, John Wiley and Sons, Inc., NY, 1987), and liposome-mediated transfection (Hawley-Nelson et al., *Focus* 15:73, 1993; Ciccarone et al., *Focus* 15:80, 1993). The production of recombinant polypeptides in cultured mammalian cells is disclosed, for example, by Levinson et al., U.S. Patent No. 4,713,339; Hagen et al., U.S. Patent No. 4,784,950; Palmiter et al., U.S. Patent No. 4,579,821; and Ringold, U.S. Patent No. 4,656,134, which are incorporated herein by reference. Suitable cultured mammalian cells include the COS-1 (ATCC No. CRL 1650), COS-7 (ATCC No. CRL 1651), BHK (ATCC No. CRL 1632), BHK 570 (ATCC No. CRL 10314), 293 (ATCC No. CRL 1573; Graham et al., J. Gen. Virol. 36:59-72, 1977) and Chinese hamster ovary (e.g. CHO-K1; ATCC No. CCL 61) cell lines. Additional suitable cell lines are known in the art and available from public depositories such as the American Type Culture Collection, Rockville, Maryland. In general, strong transcription promoters are preferred, such as promoters from SV-40 or cytomegalovirus. See,

e.g., U.S. Patent No. 4,956,288. Other suitable promoters include those from metallothionein genes (U.S. Patent Nos. 4,579,821 and 4,601,978, which are incorporated herein by reference and the adenovirus major late promoter.

Drug selection is generally used to select for cultured mammalian cells into which foreign DNA has been inserted. Such cells are commonly referred to as "transfectants". Cells that have been cultured in the presence of the selective agent and are able to pass the gene of interest to their progeny are referred to as "stable transfectants." A preferred selectable marker is a gene encoding resistance to the antibiotic neomycin. Selection is carried out in the presence of a neomycin-type drug, such as G-418 or the like. Selection systems may also be used to increase the expression level of the gene of interest, a process referred to as "amplification." Amplification is carried out by culturing transfectants in the presence of a low level of the selective agent and then increasing the amount of selective agent to select for cells that produce high levels of the products of the introduced genes. A preferred amplifiable selectable marker is dihydrofolate reductase, which confers resistance to methotrexate. Other drug resistance genes (e.g. hygromycin resistance, multi-drug resistance, puromycin acetyltransferase) can also be used.

Other higher eukaryotic cells can also be used as hosts, including insect cells, plant cells and avian cells. Transformation of insect cells and production of foreign polypeptides therein is disclosed by Guarino et al., U.S. Patent No. 5,162,222; Bang et al., U.S. Patent No. 4,775,624; and WIPO publication WO 94/06463. The use of *Agrobacterium rhizogenes* as a vector for expressing genes in plant cells has been reviewed by Sinkar et al., *J. Biosci. (Bangalore)* 11:47-58, 1987.

Fungal cells, including yeast cells, can also be used within the present invention. Yeast species of particular interest in this regard include *Saccharomyces cerevisiae*, *Pichia pastoris*, and *Pichia methanolica*. Methods for transforming Saccharomyces cells with exogenous DNA and producing recombinant polypeptides therefrom are disclosed by, for example, Kawasaki, U.S. Patent No. 4,599,311; Kawasaki et al., U.S. Patent No. 4,931,373; Brake, U.S. Patent No. 4,870,008; Welch et al., U.S. Patent No. 5,037,743; Murray et al., U.S. Patent No. 4,845,075; Kingsman et al., U.S. Patent No. 4,615,974; and Bitter, U.S. Patent No. 4,977,092. See also U.S. Patents Nos. 4,990,446; 5,063,154; 5,139,936 and 4,661,454. Transformation systems for other yeasts, including *Hansenula polymorpha*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Kluyveromyces fragilis*, *Ustilago maydis*, *Pichia pastoris*, *Pichia methanolica*, *Pichia guillermondii* and *Candida maltosa* are known in the art. See, for example, Gleeson et al., *J. Gen. Microbiol.* 132:3459-3465, 1986 and Cregg, U.S. Patent No. 4,882,279. Aspergillus cells may be utilized according to the methods of McKnight et al., U.S. Patent No. 4,935,349, which is incorporated herein by reference. Methods for transforming *Acremonium chrysogenum* are disclosed by Sumino et al., U.S. Patent No. 5,162,228, which is incorporated herein by reference. Methods for transforming Neurospora are disclosed by Lambowitz, U.S. Patent No. 4,486,533, which is incorporated herein by reference.

Prokaryotic host cells, including strains of the bacteria *Escherichia coli*, *Bacillus* and other genera are also useful host cells within the present invention. Techniques for transforming these hosts and expressing foreign DNA sequences cloned therein are well known in the art (see, e.g., Sambrook et al., ibid.). When expressing a mucin polypeptide in bacteria such as *E.*

cell, the polypeptide may be retained in the cytoplasm, typically as insoluble granules, or may be directed to the periplasmic space by a bacterial secretion sequence. In the former case, the cells are lysed, and the granules are recovered and denatured using, for example, guanidine isothiocyanate or urea. The denatured polypeptide can then be then refolded and dimerized by diluting the denaturant, such as by dialysis against a solution of urea and a combination of reduced and oxidized glutathione, followed by dialysis against a buffered saline solution. In the latter case, the polypeptide can be recovered from the periplasmic space in a soluble and functional form by disrupting the cells (by, for example, sonication or osmotic shock) to release the contents of the periplasmic space and recovering the protein, thereby obviating the need for denaturation and refolding.

Transformed or transfected host cells are cultured according to conventional procedures in a culture medium containing nutrients and other components required for the growth of the chosen host cells. A variety of suitable media, including defined media and complex media, are known in the art and generally include a carbon source, a nitrogen source, essential amino acids, vitamins and minerals. Media may also contain such components as growth factors or serum, as required. The growth medium will generally select for cells containing the exogenously added DNA by, for example, drug selection or deficiency in an essential nutrient which is complemented by the selectable marker carried on the expression vector or co-transfected into the host cell.

It is preferred to purify the polypeptides and proteins of the present invention to ≥80% purity, more preferably to ≥90% purity, even more preferably ≥95% and particularly preferred is a pharmaceutically pure state, that is greater than 99.9% pure with respect to

contaminating macromolecules, particularly other proteins and nucleic acids, and free of infectious and pyrogenic agents. Preferably, a purified protein is substantially free of other proteins, particularly other proteins of animal origin.

Zvegf2 polypeptides and proteins are purified by conventional protein purification methods, typically by a combination of chromatographic techniques. Polypeptides and proteins comprising a polyhistidine affinity tag (typically about 6 histidine residues) are purified by affinity chromatography on a nickel chelate resin. See, for example, Houchuli et al., *Bio/Technol.* 6: 1321-1325, 1988.

Zvegf2 can also be used to identify inhibitors of its activity. Samples can be tested for inhibition of zvegf2 activity within a variety of assays designed to measure receptor binding or the stimulation/inhibition of zvegf2-dependent cellular responses. For example, zvegf2-responsive cell lines can be transfected with a reporter gene construct that is responsive to a zvegf2-stimulated cellular pathway. Reporter gene constructs of this type are known in the art, and will generally comprise a zvegf2-activated serum response element (SRE) operably linked to a gene encoding an assayable protein, such as luciferase. Candidate compounds, solutions, mixtures or extracts are tested for the ability to inhibit the activity of zvegf2 on the target cells as evidenced by a decrease in zvegf2 stimulation of reporter gene expression. Assays of this type will detect compounds that directly block zvegf2 binding to cell-surface receptors, as well as compounds that block processes in the cellular pathway subsequent to receptor-ligand binding. In the alternative, compounds or other samples can be tested for direct blocking of zvegf2 binding to receptor using zvegf2 tagged with a detectable label (e.g., ^{125}I, biotin, horseradish peroxidase, FITC,

and the like). Within assays of this type, the ability of a test sample to inhibit the binding of labeled zvegf2 to the receptor is indicative of inhibitory activity. Receptors used within such assays may be cellular receptors or isolated, immobilized receptors. Within a third type of assay, inhibition of zvegf2 mitogenic activity is measured. Such activity is detected as a decrease in [3H]-thymidine incorporation after addition of the test sample to an assay system as disclosed above. A preferred target cell type for use in mitogenesis assays is human dermal fibroblasts.

Zvegf2 proteins can be used therapeutically to stimulate the revascularization of tissue or the re-endothelialization of vascular tissue. Specific applications include, without limitation, the treatment of full-thickness skin wounds, including venous stasis ulcers and diabetic ulcers; treatment of burns; skin grafting; to promote the growth of tissue damaged by periodontal disease; to promote endothelialization of vascular grafts and stents; and to promote vessel repair and development of collateral circulation following myocardial infarction. The proteins are also useful additives in tissue adhesives for promoting revascularization of the healing tissue.

For pharmaceutical use, the zvegf2 polypeptides and proteins are formulated for topical or parenteral, particularly intravenous or subcutaneous, delivery according to conventional methods. Intravenous administration will be by bolus injection or infusion over a typical period of one to several hours. In general, pharmaceutical formulations will include a zvegf2 polypeptide or protein in combination with a pharmaceutically acceptable vehicle, such as saline, buffered saline, 5% dextrose in water or the like. Formulations may further include one or more excipients, preservatives, solubilizers, buffering agents, albumin to

prevent protein loss on vial surfaces, etc. Methods of formulation are well known in the art and are disclosed, for example, in *Remington's Pharmaceutical Sciences*, Gennaro, ed., Mack Publishing Co., Easton PA, 1990, which is incorporated herein by reference. Zvegf2 will generally be used in a concentration of about 10 to 100 µg/ml of total volume, although concentrations in the range of 1 ng/ml to 1000 µg/ml may be used. For topical application, such as for the promotion of wound healing, the protein will be applied in the range of 0.1-10 µg/cm² of wound area, with the exact dose determined by the clinician according to accepted standards, taking into account the nature and severity of the condition to be treated, patient traits, etc. Determination of dose is within the level of ordinary skill in the art. The therapeutic formulations may be administered for acute treatment, over one week or less, often over a period of one to three days or may be used in chronic treatment over several months or years. In general, a therapeutically effective amount of zvegf2 is an amount sufficient to produce a clinically significant change in the treated condition, such as a clinically significant reduction in time required by wound closure, a significant reduction in wound area, or a significantly increased histological score.

The zvegf2 proteins of the present invention are also useful within the laboratory field for promoting the growth of mesenchymal cells (including fibroblasts and smooth muscle cells) in culture. The polypeptides are added to cell culture media at a concentration of about 10 pg/ml to about 100 ng/ml. Those skilled in the art will recognize that Zvegf2 proteins can be advantageously combined with other growth factors in culture media.

Zvegf2 polypeptides can also be used to prepare antibodies that specifically bind to zvegf2 polypeptides.

As used herein, the term "antibodies" includes polyclonal antibodies, monoclonal antibodies, antigen-binding fragments thereof such as $F(ab')_2$ and Fab fragments, single chain antibodies, and the like, including genetically engineered antibodies. Non-human antibodies may be humanized by grafting only non-human CDRs onto human framework and constant regions, or by incorporating the entire non-human variable domains (optionally "cloaking" them with a human-like surface by replacement of exposed residues, wherein the result is a "veneered" antibody). In some instances, humanized antibodies may retain non-human residues within the human variable region framework domains to enhance proper binding characteristics. Through humanizing antibodies, biological half-life may be increased, and the potential for adverse immune reactions upon administration to humans is reduced. One skilled in the art can generate humanized antibodies with specific and different constant domains (i.e., different Ig subclasses) to facilitate or inhibit various immune functions associated with particular antibody constant domains. Alternative techniques for generating or selecting antibodies useful herein include *in vitro* exposure of lymphocytes to zvegf2 protein or polypeptide, and selection of antibody display libraries in phage or similar vectors (for instance, through use of immobilized or labeled zvegf2 protein or polypeptide). Antibodies are defined to be specifically binding if they bind to a zvegf2 polypeptide or protein with an affinity at least 10-fold greater than the binding affinity to control (non-zvegf2) polypeptide or protein. The affinity of a monoclonal antibody can be readily determined by one of ordinary skill in the art (see, for example, Scatchard, *Ann. NY Acad. Sci.* 51: 660-672, 1949).

Methods for preparing polyclonal and monoclonal antibodies are well known in the art (see, for example,

Hurrell, J. G. R., Ed., *Monoclonal Hybridoma Antibodies: Techniques and Applications*, CRC Press, Inc., Boca Raton, FL, 1982, which is incorporated herein by reference). As would be evident to one of ordinary skill in the art, polyclonal antibodies can be generated from a variety of warm-blooded animals such as horses, cows, goats, sheep, dogs, chickens, rabbits, mice, and rats. The immunogenicity of a zvegf2 polypeptide may be increased through the use of an adjuvant such as alum (aluminum hydroxide) or Freund's complete or incomplete adjuvant. Polypeptides useful for immunization also include fusion polypeptides, such as fusions of a zvegf2 polypeptide or a portion thereof with an immunoglobulin polypeptide or with maltose binding protein. The polypeptide immunogen may be a full-length molecule or a portion thereof. If the polypeptide portion is "hapten-like", such portion may be advantageously joined or linked to a macromolecular carrier (such as keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA) or tetanus toxoid) for immunization.

A variety of assays known to those skilled in the art can be utilized to detect antibodies which specifically bind to zvegf2 polypeptides. Exemplary assays are described in detail in *Antibodies: A Laboratory Manual*, Harlow and Lane (Eds.), Cold Spring Harbor Laboratory Press, 1988. Representative examples of such assays include: concurrent immunoelectrophoresis, radio-immunoassays, radio-immunoprecipitations, enzyme-linked immunosorbent assays (ELISA), dot blot assays, Western blot assays, inhibition or competition assays, and sandwich assays.

Antibodies to zvegf2 may be used for affinity purification of the protein, within diagnostic assays for determining circulating levels of the protein; for detecting or quantitating soluble zvegf2 polypeptide as a marker of underlying pathology or disease; for

immunolocalization within whole animals or tissue sections, including immunodiagnostic applications; for immunohistochemistry; and as antagonists to block protein activity *in vitro* and *in vivo*. Antibodies to zvegf2 may also be used for tagging cells that express zvegf2; for affinity purification of zvegf2 polypeptides and proteins; in analytical methods employing FACS; for screening expression libraries; and for generating anti-idiotypic antibodies. Antibodies can be linked to other compounds, including therapeutic and diagnostic agents, using known methods to provide for targetting of those compounds to cells expressing receptors for zvegf2. For certain applications, including *in vitro* and *in vivo* diagnostic uses, it is advantageous to employ labeled antibodies. Suitable direct tags or labels include radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent markers, chemiluminescent markers, magnetic particles and the like; indirect tags or labels may feature use of biotin-avidin or other complement/anti-complement pairs as intermediates. Antibodies of the present invention may also be directly or indirectly conjugated to drugs, toxins, radionuclides and the like, and these conjugates used for *in vivo* diagnostic or therapeutic applications.

Inhibitors of zvegf2 activity (zvegf2 antagonists) include anti-zvegf2 antibodies and soluble zvegf2 receptors, as well as other peptidic and non-peptidic agents (including ribozymes). Such antagonists can be use to block the mitogenic, chemotactic, or angiogenic effects of zvegf2. These antagonists are therefore useful in reducing the growth of solid tumors by inhibiting neovascularization of the developing tumor or by directly blocking tumor cell growth; in the treatment of diabetic retinopathy, psoriasis, arthritis, and scleroderma; and in reducing fibrosis, including scar formation. In view of the mitogenic activity of zvegf2

to intimal smooth muscle cells. Inhibitors may also be useful in the treatment of proliferative vascular disorders, including atherosclerosis and intimal hyperplastic restenosis following angioplasty, endarterectomy, vascular grafting, organ transplant, or vascular stent emplacement. In addition to anti-zvegf2 antibodies, inhibitors useful in this regard include small molecule inhibitors and angiogenically or mitogenically inactive receptor-binding fragments of zvegf2 polypeptides. Inhibitors are formulated for pharmaceutic use as generally disclosed above, taking into account the precise chemical and physical nature of the inhibitor and the condition to be treated. The relevant determinations are within the level of ordinary skill in the formulation art.

Polynucleotides encoding zvegf2 polypeptides are useful within gene therapy applications where it is desired to increase or inhibit zvegf2 activity. For example, Isner et al., *The Lancet* (ibid.) reported that VEGF gene therapy promoted blood vessel growth in an ischemic limb. Additional applications of zvegf2 gene therapy include stimulation of wound healing and repopulation of vascular grafts. Antisense methodology can be used to inhibit zvegf2 gene trascription, such as to inhibit cell proliferation *in vivo*.

The invention is further illustrated by the following non-limiting examples.

Examples

Example 1

A cDNA library was prepared from human heart RNA using a Marathon™ cDNA Amplification Kit (Clontech Laboratories, Inc., Palo Alto, CA). This cDNA was used as template to generate DNA encoding human zvegf2. PCR primers were designed from the sequence of an expressed sequence tag ("EST") in a DNA sequence database. Five nl

of a 1:100 dilution of template DNA was combined with 20 pmoles of each primer (ZC10917, SEQ ID NO:4; ZC10924, SEQ ID NO:5) in a PCR mixture. The reaction mixture was incubated at 94°C for 1 minute, then run for 35 cycles of 94°C, 20 seconds; 68°C, 1 minute; followed by an extension at 74°C for 10 minutes. The PCR product was purified by gel electrophoresis in duplicate samples. One sample was extracted from the gel using a Qiaquick™ column (Qiagen Inc., Chatsworth, CA) for subsequent use as a probe for Northern blots. The other sample was extracted from the gel using a commercially available kit (Wizard™ kit; Promega Corp., Madison, WI) and sequenced. The sequence matched that of the EST.

The remainder of the zvegf2 coding sequence was cloned by RACE (rapid amplification of cDNA ends) essentially as disclosed in the *Marathon™ cDNA Amplification Kit Protocol and Reference Manual* (Clontech Laboratories, Inc.) using primers complementary to the EST sequence. A 5' RACE product was amplified from the heart cDNA library using 5 µl of a 1:100 dilution of template DNA and 20 pmoles each of primers ZC10920 (SEQ ID NO:6) and AP1 (obtained from Clontech Laboratories). The reaction mixture was incubated at 94°C for one minute, then run for 35 cycles of 94°C, 20 seconds; 68°C, 4 minutes; followed by an extension at 74°C for 10 minutes. The 3' RACE product was amplified from the same library using 5 µl of a 1:100 dilution of template DNA and 20 pmoles each of primers ZC10913 (SEQ ID NO:7) and AP1. Reaction conditions were the same as for the 5' RACE. Nested primers were used for further characterization of the resulting products. The 5' and 3' RACE products were reamplified using 20 pmoles each of ZC10918 (SEQ ID NO:8) and AP2 (obtained from Clontech Laboratories), and ZC10923 (SEQ ID NO:9) and AP2, respectively. The reaction mixtures were incubated at 94°C for one minute, then run for 34 cycles of 94°C, 20 seconds; 68°C, 4

minutes; followed by a 74°C incubation for 10 minutes. The reaction products were 1.5 kb and 0.8 kb for the 5' and 3' reactions, respectively. Gel electrophoresis showed the 5' and 3' nested RACE products to be 1.3 kb and 0.8 kb, respectively. DNA was extracted from a gel slice using a commercially available kit (Wizard™ kit; Promega Corp.) and sequenced.

Analysis of the DNA sequence (SEQ ID NO:1) and the encoded polypeptide (SEQ ID NO:2) indicated the presence of a 1062 nucleotide open reading frame encoding a putative signal sequence of 21 to 23 amino acid residues, a putative propeptide cleavage site at residues 108 to 109, a Balbiani ring motif (residues 257 to approximately 274), and one slightly degenerate Balbiani ring motif (approximately residues 275 to 294). The DNA further included a polyadenylation signal and poly(A) tail.

Example 2

A full-length zvegf2 DNA was generated by PCR using a the heart cDNA library (5 µl of a 1:100 dilution) as template and 20 pmoles each of primers ZC11782 (SEQ ID NO:10) and ZC11783 (SEQ ID NO:11). The reaction mixture was incubated at 94°C for 1 minute, then for 26 cycles of 94°C, 30 seconds; 70°C, 3 minutes; then incubated at 74°C for 10 minutes. The resulting 1,073 bp fragment was cut with BamHI and KpnI, gel purified on a 0.7% agarose gel, and subcloned into pOZ-1, which had been cut with KpnI and BamHI. Plasmid pOZ-1 is a mammalian cell expression vector comprising the mouse metallothionein-1 promoter; the bacteriophage T7 promoter flanked by multiple cloning sites containing unique restriction sites for insertion of coding sequences; the human growth hormone terminator; the bacteriophage T7 terminator; an E. coli origin of replication; a bacterial beta lactamase gene; a mammalian selectable marker expression unit comprising the SV40

promoter and origin, a DHFR gene, and the SV40 transcription terminator; and a sequence encoding a C-terminal polyhistidine tag downstream of the MT-1 promoter. The resulting vector, designated zvegf2-FL, was sequenced and found to have the correct sequence encoding a His-tagged zvegf2.

A DNA construct encoding a His-tagged, truncated zvegf2 polypeptide was also constructed. The encoded polypeptide consisted of residues 1 to 137 of SEQ ID NO:2 with 6 histidine residues attached to the carboxyl terminus. The truncated zvegf2 sequence was generated by PCR using the 5' RACE product disclosed in Example 1 as template. The DNA was diluted 1:100, a 5 µl of this template was combined with 20 pmoles each of primers ZC11626 (SEQ ID NO:12) and ZC11627 (SEQ ID NO:13). The reaction mixture was incubated at 94°C for one minute, then run for 4 cycles of 94°C, 20 seconds; 62°C, 3 minutes; 23 cycles of 94°C, 20 seconds; 70°C, 3 minutes; followed by a 10 minute incubation at 74°C. The resulting 601 bp fragment was cut with KpnI and BamHI and purified by electrophoresis on a 1% agarose gel. The resulting fragment was ligated with vector pOZ-1. The resulting vector, designated zvegf2-T, was sequenced, revealing the presence of two silent nucleotide substitutions in the zvegf2 sequence. In this construct, nucleotide 297 of SEQ ID NO:1 (A) was replaced with G, and nucleotide 549 (T) was replaced with C.

BHK cells were transfected with the zvegf2-FL and zvegf2-T constructs, and with an unrelated negative control plasmid. Transfection pools were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum. Cultures reaching 50% confluence were washed once with serum-free medium (DMEM with 5 ng/ml selenium, 5 µg/ml transferrin, 0.5 µg/ml fetuin, and 0.25 µg/ml insulin) and incubated in the same medium for 36 hours. The resulting conditioned media were concentrated

100-fold on 5,000 molecular weight cut-off filters (Millipore Ultrafree-15), and 8 µl of each of the resulting samples was subjected to SDS-PAGE under reducing conditions (Novex precast Nupage 4-12% acrylamide gels run with MES buffer). As a positive control, purified polyhistidine-tagged MPL receptor (disclosed in WIPO Publication WO 95/21920) was loaded in quantities of 1000 ng, 100 ng, and 10 ng. Gels were blotted to nitrocellulose filters and probed with a mouse monoclonal antibody specific for a C-terminal oligohistidine tag (Invitrogen cat. #R930-25). Blots were probed with secondary antibody specific to mouse IgG conjugated to horseradish peroxidase (Santa Cruz Biotech cat. # sc-2005). His-tagged protein was visualized using chemiluminescent substrate (Pierce Chemical Co., cat. # 34075). A major protein band migrating at 25 kDa and a minor band migrating at 50 kDa were detected in the zvegf2-FL conditioned media samples, but not in conditioned media from zvegf2-T or control transfected cells. By comparing the signal intensity of these bands relative to the MPL receptor positive control, the quantity of vegf2 was estimated to be approximately 50 ng, which corresponded to 60 ng/ml of secreted protein in the conditioned media. See Fig. 2. The data are consistent with c-terminal processing of the zvegf2 polypeptide at residues 205-206 of SEQ ID NO:2.

Example 3

Human multiple tissue Northern blots (I, III, and IV from Clontech Laboratories) were probed to determine the tissue distribution of zvegf2. The PCR product disclosed in Example 1 was labeled with P using a commercial kit (Multiprime™ DNA labeling system; Amersham Corp.). Unincorporated radioactivity was removed with a push column (NucTrap® probe purification column; Stratagene Cloning Systems, LaJolla, CA). The

multiple tissue blots were prehybridized for 3 hours at 68°C with ExpressHyb™ hybridization solution (Clontech Laboratories). 54 µl (7 x 10⁷ cpm) of labeled zvegf2 probe was boiled for 5 minutes, placed on ice 1 minute, then added to 7 ml of ExpressHyb™ hybridization solution. The solution was mixed and added to the blots. Hybridization was carried out overnight at 68°C. The blots were then washed for 40 minutes at room temperature in several changes of 2 x SSC, 0.05% SDS, then once in 0.1 x SSC, 0.1% SDS for 40 minutes at 50°C. The washed blots were exposed to film overnight at -80°C. Heart, uterus, and small intestine showed high expression of zvegf2 mRNA. Skeletal muscle, lung, colon, and spleen showed lower levels. The transcript size was approximately 2.5 kb.

Example 4

The human zvegf2 gene locus was mapped to the Xp22.3 - p22.1 region of the X chromosome using fluorescence *in situ* hybridization.

To prepare a probe the following were added to a 1.5 ml microcentrifuge tube on ice: 1 µg of a P1 genomic clone (Sternberg, *TIG* 8:11-15, 1992) containing the human zvegf-2 gene; 5 µl 10 x nick translation buffer (0.5 M Tris/HCl, 50 mM $MgCl_2$, 0.5 mg/ml BSA (nuclease free)); 5 µl dNTPs solution containing 0.5 mM dATP, 0.5 mM dGTP, and 0.5 mM dCTP; 5 µl 5 mM Bio-11-dUTP; 5 µl 100 mM DTT; 5 µl DNase I (a 1000 x dilution from a 10 U/µl stock, RNase-free, Boehringer Mannheim, Indianapolis, IN); 2.5 µl DNA polymerase I (5 U/µl, Boehringer Mannheim); and distilled H_2O to a final volume of 50 µl. After mixing, the reaction was incubated at 15°C for 1 hr in a microcooler (Boekel, Feasterville, PA). The reaction was stopped by adding 5 µl 0.5 M EDTA, pH 7.4 to the mixture. The probe was purified using a G-50 DNA purification spin column according to the manufacturer's

instructions (Worthington Biochemical Corporation, Freehold, NJ).

Metaphase chromosomes were obtained from a HEL cell culture. 100 µl colcemid (10 µg/ml stock, GIBCO BRL, Gaithersburg, MD) was added to the medium of a 100 x 15 mm petri dish used for the cell culture and incubated at 37°C for 2.5 - 3 hours, then the medium was removed from the petri dish using a 10 ml sterile plastic pipette and transferred to a 15 ml conical tube (Blue Max™; Becton Dickinson, Bedford, MA). Two ml of 1 x PBS (140 mM NaCl, 3 mM KCL, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2) was added to the petri dish for rinsing using a 5 ml sterile plastic pipette, then transferred to the conical tube. Two ml of trypsin (stock solution, GIBCO BRL) was added to the petri dish using a sterile 5 ml plastic pipette, and the petri dish was gently rocked and put into a 37°C incubator for 3 - 5 minutes. The cells were then washed from the petri dish using a 5 ml sterile plastic pipette and added to the tube with the medium. The culture tube was centrifuged at 1100 rpm for 8 minutes, and all but 0.5 ml of the supernatant was removed. The pellet was resuspended by tapping, then 8 ml of 0.075 M KCl (prewarmed to 37°C) was added slowly and gently. The suspension was mixed gently and placed in a 37°C water bath for 10 minutes. After the incubation the suspension was centrifuged at 1100 rpm for 5 minutes, and all but 0.5 ml of the supernatant above the pellet was aspirated off. The pellet was resuspended by tapping the tube. Cold methanol:acetic acid (3:1) was added dropwise with shaking to fix the cells. Two ml of fix was added in this manner. A total of 8 ml was added slowly and gently. The tube was placed in a refrigerator for 20 minutes, then centrifuged for 8 minutes at 1100 rpm. The supernatant was again aspirated off, and the fixation process was repeated two more times. To drop metaphase spreads on 2s x 75 mm precleaned, frosted glass slides

(VWR, Seattle, WA), 5 µl of 80% acetic acid was spotted on each slide with a 20 µl micropipette (Gilson International, Middleton, WI), followed by 5 µl of the cell suspension. The slides were allowed to air dry, then aged overnight in a 42°C oven (Boekel) before use. The slides were scored for suitable metaphase spreads using a microscope equipped with a phase contrast condenser. Unused metaphase chromosome slide preparations were stored at -70°C.

Hybridization mixtures were then prepared. For each slide, 2.5 - 5 µg competitor DNA (Cot-1 DNA, GIBCO BRL), 60 - 200 ng biotin-labeled P1 DNA containing the mvegfl gene, 50 - 100 µg carrier DNA denatured salmon testes DNA, Sigma Chemical Co., St. Louis, MO), 1 µl 3 M Na acetate and 2 volumes ethanol were placed in a 1.5 ml sterile microcentrifuge tube and vacuum-dried in a speed-vac concentrator. The resulting pellet was dissolved in 10 µl of a hybridization solution containing 10% dextran sulfate, 2 x SSC and 50 - 65% formamide (EM Science, Gibbstown, NJ). The probe and competitor DNA were denatured at 70 - 80°C for 5 minutes, chilled on ice, and pre-annealed at 37°C for 1 - 2 hours. In some cases, a digoxigenin-labeled centromeric probe specific to the X chromosome (DXZ1, Oncor, Gaithersburg, MD) was added to the hybridization mixture after the pre-annealing step.

Denaturation of the chromosomes was done by immersion of each slide in 70% formamide, 2 x SSC at 70 - 80°C for 5 minutes, followed by immediate cooling in ice-cold 70% ethanol and then 100% ethanol for 5 - 10 minutes each. The slides were then air dried and warmed to 42°C just before pipetting the hybridization mixtures onto them with a 20 µl micropipette. The hybridization mixture and chromosomes were then covered with a 18 x 18 mm, Number 1 coverslip (VWR). The hybridizations proceeded in a moist chamber overnight at 37°C.

After removal of the coverslips, the slides were washed 3 x 5 minutes per wash in 50 - 65% formamide, 2 x SSC at 42°C; 3 x 5 minutes in 2 x SSC at 42°C, and once for 3 minutes in 4 x SSC, 1.05% polyoxyethylenesorbitan monolaurate (Tween-20; Sigma Chemical Co.). Washing was followed by a 20 minute preincubation with 4 x SSC containing 5% non-fat dry milk (Carnation, Los Angeles, CA) in a moist chamber (100 µl under a 24 x 50 mm coverslip). The posthybridization steps proceeded then with a 20 minute incubation with fluorescein avidin DCS (cell sorter grade, Vector, Burlingame, CA) (100 µl, 5 µg/ml, in 4 x SSC, 5% non-fat dry milk under a 24 x 50 mm coverslip). The slides were then washed 3 x 3 minutes in 4 x SSC, 0.05% polyoxyethylenesorbitan monolaurate, followed by a 20 minute incubation with biotinylated goat anti-avidin D (affinity purified, Vector) (5 µg/ml in 4 x SSC, 5% non-fat dry milk under a 24 x 50 mm coverslip). The slides were again washed 3 x 3 minutes in 4 x SSC, 0.05% polyoxyethylenesorbitan monolaurate, followed by another incubation with fluorescein avidin DCS (100 µl/ml in 4 x SSC, 5% non-fat dry milk under a 24 x 50 mm coverslip). In some cases, the signal amplification procedure was repeated one additional time. For the preparations which included the X chromosomal DXZ1 centromeric probe, a 1:100 dilution of biotin-labeled mouse anti-digoxin (Sigma Chemical Co.) was included in the first incubation with biotinylated goat anti-avidin D. The final washes were for 2 x 3 minutes in 4 x SSC, 0.05% Tween-20; and 1 x 3 minutes in 1 x PBS. The slides were mounted in antifade medium (4 parts glycerol containing; 2' 1,4-diazobicyclo-(2,2,2) octane (DABCO, Sigma Chemical Co.) and one part 0.2 M Tris-HCl, pH 7.5 and 0.25 - 0.5 µg/ml propidium iodide). The slides were viewed on an Olympus BH2 microscope equipped with a BH2-RFC reflected light fluorescence attachment, a PM-10 ADS automatic

photomicrographic system, an Optronics ZVS-47E CCD RGB color video camera system and a FITC/Texas Red filter set for FITC visualization. Images of the metaphase chromosome spreads were digitized and stored using the Optronics video imaging camera system and Optimus software (Bothell, WA) running on a 486 computer.

Positive labeling was seen only on the p arm of the X chromosome (n > 50 metaphase spreads). Twenty-three chromosomes were chosen for subchromosomal mapping. Using the FIpter method (Lichter et. al., *Science* 247: 64-69, 1990), 44 hybridization signals were considered suitable for measurement, of which 95.5% were localized to the Xp22.3 - p22.1 chromosomal region.

Example 5

Zvegf2 protein is analyzed for mitogenic activity on human dermal fibroblasts (SK-5). SK-5 cells are plated at a density of 8,000 cells/well in 24-well culture plates and grown for approximately 72 hours in DMEM containing 10% fetal calf serum at 37°C. The cells are made quiescent by incubating them for 24 hours in serum-free DMEM/Hams F-12 containing insulin (5μg/ml), transferrin (20 μg/ml), selenium (16 pg/ml) and 0.1% bovine serum albumin (ITS medium). At the time of the assay, the ITS medium is removed, and test samples (conditioned media from BHK cells transfected with plasmid zvegf2-FL) or control samples (conditioned media from BHK cells transfected with an SRE-luciferase contruct or from untransfected BHK-570 cells) are added to the wells in triplicate. Media are concentrated 10X fold using a PM membrane, then diluted either 50 or 10 fold with ITS medium and added to the test wells. After another 24 hour incubation, mitogenic activity is assessed by uptake of [^{3}H]-thymidine. For measurement of [^{3}H]-thymidine incorporation, 50 μl of a 20 μCi/ml stock in DMEM is added directly to the cells, for a final

activity of 1 μCi/well. The cells are subsequently incubated for 4 hours at 37°C, washed once with PBS, and incubated with 0.25 ml of trypsin until cells detach. The cells are harvested using a Filtermate™ harvester (Packard Instrument Co., Meriden, CT) onto 24-well filter plates. Subsequently, the plates are dried at 80°C for 30 minutes, sealed after adding 250 μl/well Microscin-O™ (Packard Instrument Co.) and counted on a Topcount™ microplate scintillation counter (Packard Instrument Co.).

Example 6

Culture medium was conditioned for 48 hours in the presence of BHK cells transfected with the full-length zvegf2 construct (zvegf2-FL). One liter of conditioned medium was passed through a 0.2 micron filter, then adjusted to 20 mM imidazole, 410 mM NaCl, and pH 8.0 with NaOH. The adjusted medium was passed over a 15 ml column of nickel chelate resin (Ni-NTA agarose; Qiagen, Chatsworth, CA). The column was washed extensively with phosphate buffered saline (360 mM NaCl, 8.1 mM KCl, 30 mM phosphate pH 8.0) containing 20 mM imidazole, followed by phosphate buffered saline containing 100 mM imidazole. Bound protein was eluted with 15 ml phosphate buffered saline containing 200 mM imidazole.

The eluent was concentrated 300 X on a 3 kD cut-off filter, washed with phosphate buffered saline, and concentrated to a 30 μl volume on the same cut-off filter. 1.75 μl of the resulting concentrate was analyzed by electrophoresis on a 4-12% SDS-polyacrylamide gel in the presence of 3% β-mercaptoethanol, followed by Coomassie blue staining. Three protein bands, migrating at 26, 28, and 50 kD, were detected.

An identical gel was blotted to a PVDF membrane using a NOVEX II blot module (Novex, San Diego, CA).

Individual protein bands were cut from the blot and sequenced using an Applied Biosystems 476A protein sequencer equipped with on-line high performance liquid chromatography. The amino terminal sequence of the 26 kD band was determined to be SIQIPEEDR, which corresponds to the predicted sequence of zvegf2 starting at amino acid 206. The amino terminal sequence of both the 28 and 50 kD bands was determined to be XXNEHGPVKRXXQ, which corresponds to the predicted sequence of zvegf2 starting at amino acid 22.

The zvegf2(22-362) polypepeptide has a predicted polypeptide backbone molecular mass of 38,000, suggesting that the 50 kD Coomassie-stained band corresponds to zvegf2(22-362). The two bands migrating at 26 and 28 kD appeared to result from cleavage of this 50 kDa protein at residue 206 (Ser). The resulting two peptides, zvegf2(22-205) and zvegf2(206-362), have predicted molecular masses of 20,900 and 17,000 respectively. Furthermore, only the 26 kD and 50 kD Coomassie stained bands cross-react by Western blot with a monoclonal antibody (Invitrogen cat. R930-25) directed against the 6 histidine C-terminal tag. The presence of a non-his-tagged peptide in the nickel-purified zvegf2 product can likely be attributed to disulfide interactions between zvegf2 polypeptides.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: ZymoGenetics, Inc
1201 Eastlake Avenue East
Seattle, Washington 98102
United States of America

(ii) TITLE OF THE INVENTION: VASCULAR ENDOTHELIAL GROWTH FACTOR

(iii) NUMBER OF SEQUENCES: 14

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: ZymoGenetics, Inc.
(B) STREET: 1201 Eastlake Avenue East
(C) CITY: Seattle
(D) STATE: WA
(E) COUNTRY: USA
(F) ZIP: 98102

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ for Windows Version 2.0

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA
(A) APPLICATION NUMBER:
(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Parker, Gary E
(B) REGISTRATION NUMBER: 31,648
(C) REFERENCE/DOCKET NUMBER: 96-15PC

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 206-443-6673
(B) TELEFAX: 206-442 6678
(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1197 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(ix) FEATURE

(A) NAME/KEY: Coding Sequence
(B) LOCATION: 7...1068
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GGTACC ATG TAC AGA GAG TGG GTA GTG GTG AAT GTT TTC ATG ATG TTG
48
Met Tyr Arg Glu Trp Val Val Val Asn Val Phe Met Met Leu
1 5 10

TAC GTC CAG CTG GTG GAG GCC TCC ACT AAT GAA CAT GGA CCA GTG AAG
96
Tyr Val Gln Leu Val Glu Ala Ser Thr Asn Glu His Gly Pro Val Lys
15 20 25 30

GAA TCA TTT GAG TCC AGA TTG GAA CGA GAA TTA GAA GAA ATC ATT GTT
144
Arg Ser Phe Glu Ser Thr Leu Glu Arg Glu Leu Glu Glu Ile Val Leu
35 40 45

GCT TCT AGT TTG GAG GAA CTA CTT CGA ATT ACT CAC TCT GGG GAC TGG
192
Ala Ser Ser Leu Glu Glu Leu Leu Arg Ile Thr His Ser Gly Asp Trp
50 55 60

AAG CTG TGG AGA TGC AGC CTG AGG CTC AAA ACT TTT AGC AGT ATG GAC
240
Lys Leu Trp Arg Cys Arg Leu Arg Leu Lys Ser Phe Thr Ser Met Asp
65 70 75

TAT CGC TCA GCA TCC CAT CGG TCC ACT AGG TTT GCG GCA ACT TTC TAT
288
Ser Arg Ser Ala Ser His Arg Ser Thr Arg Phe Ala Ala Thr Phe Tyr
80 85 90

GAC ATT GAA AGA CTA AAA GTT ATA GAT GAA GAA TGG CAA AGA ACT CAG
336
Asp Ile Glu Thr Leu Lys Val Ile Asp Glu Glu Trp Gln Arg Thr Gln
95 100 105 110

TGC AGC CCT AGA GAA ACG TGC GTG GAG GTG GCC AGT GAG CTG GGG AAG
384
Cys Ser Pro Arg Glu Thr Cys Val Glu Val Ala Ser Glu Leu Gly Lys
115 120 125

AGT ACC AAC ACA TTC TTC AAG CCC CCT TGT GTG AAC GTG TTC CGA TGT
432
Ser Thr Asn Thr Phe Phe Lys Pro Pro Cys Val Asn Val Phe Arg Cys
130 135 140

GGT GGC TGT TGC AAT GAA GAG AGC CTT ATC TGT ATG AAC ACC AGC ACC
480
Gly Gly Cys Cys Asn Glu Glu Ser Leu Ile Cys Met Asn Thr Ser Thr
145 150 155

TCG TAC ATT TCC AAA CAG CTC TTT GAG ATA TCA GTG CCT TTG ACA TCA
528
Ser Tyr Ile Ser Lys Gln Leu Phe Glu Ile Ser Val Pro Leu Thr Ser
160 165 170

[illegible]
576
Val Pro Glu Leu Val Pro Val Lys Val Ala Asn His Thr Gly Cys Lys
175 180 185 190

```
                                                                  CAT CCA TAC TCA ATT ATC AGA ACA TCC
724
 Cys Leu Pro Thr Ala Pro Arg His Pro Tyr Ser Ile Ile Arg Arg Ser
                     135                 140                 145

ATC GAG ATC CCT GAA GAA GAT CGC TGT TCC CAT TCC AAG AAA CTC TGT
772
 Ile Glu Ile Pro Glu Glu Asp Arg Cys Ser His Ser Lys Lys Leu Cys
             150                 155                 160

CCT ATT GAC ATG CTA TGG GAT AGC AAC AAA TGT AAA TGT GTT TTG GAG
820
 Pro Ile Asp Met Leu Trp Asp Ser Asn Lys Cys Lys Cys Val Leu Gln
         165                 170                 175

GAG GAA AAT CCA CTT GCT GGA ACA GAA GAC CAC TCT CAT CTC CAG GAA
868
 Glu Glu Asn Pro Leu Ala Gly Thr Glu Asp His Ser His Leu Gln Glu
     180                 185                 190

CCA GCT CTC TGT GGG CCA CAC ATG ATG TTT GAC GAA GAT CGT TGC GAG
916
 Pro Ala Leu Cys Gly Pro His Met Met Phe Asp Glu Asp Arg Cys Glu
 195                 200                 205

TGT GTC TGT AAA ACA CCA TGT CCC AAA GAT CTA ATC CAG CAC CCC AAA
964
 Cys Val Cys Lys Thr Pro Cys Pro Lys Asp Leu Ile Gln His Pro Lys
             210                 215                 220

AAC TGC AGT TGC TTT GAG TGC AAA GAA AGT CTG GAG ACC TGC TGC CAG
1012
 Asn Cys Ser Cys Phe Glu Cys Lys Glu Ser Leu Glu Thr Cys Cys Gln
         225                 230                 235

AAG CAC AAG CTA TTT CAC CCA GAC ACC TGC AGC TGT GAG GAC AGA TGC
1060
 Lys His Lys Leu Phe His Pro Asp Thr Cys Ser Cys Glu Asp Arg Cys
     240                 245                 250

CCA TTT CAT ACT AGA CCA TGT GAA AGT GGC AAA TGT GAA CCA AGA TGT
1108
 Pro Phe His Thr Arg Pro Cys Glu Ser Gly Lys Cys Glu Pro Arg Cys
 255                 260                 265
```

[illegible] AAG GAG AAA AGG GCT GCC CAG GGG CCC CAC AGC
1056
His Lys Asn Phe Pro Lys Glu Lys Arg Ala Ala Gln Gly Pro His Ser
335 340 345 350

TGA AAG AAT CCT GGATCCGGTG GCCATCACCA TCACCATCAC TAACTCGAG
1107
Arg Lys Asn Pro

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 354 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Tyr Arg Glu Trp Val Val Val Asn Val Phe Met Met Leu Tyr Val
1 5 10 15
Gln Leu Val Gln Gly Ser Ser Asn Glu His Gly Pro Val Lys Arg Ser
20 25 30
Ser Gln Ser Thr Leu Glu Arg Ser Glu Gln Gln Ile Arg Ala Ala Ser
35 40 45
Ser Leu Glu Glu Leu Leu Arg Ile Thr His Ser Glu Asp Trp Lys Leu
50 55 60
Trp Arg Cys Arg Leu Arg Leu Lys Ser Phe Thr Ser Met Asp Ser Arg
65 70 75 80
Ser Ala Ser His Arg Ser Thr Arg Phe Ala Ala Thr Phe Tyr Asp Ile
85 90 95
[illegible] Val Ile [illegible] Thr [illegible] Cys Ser
100 105 110
[illegible] Ser Thr
115 120 125
[illegible] Phe Arg [illegible]
130 135 140
[illegible] Glu Ser Leu Ile Cys Met Asn Thr Ser Thr Ser [illegible]
145 150 155 160

```
Ile Ser Lys Gln Leu Phe Glu Ile Ser Val Pro Leu Thr Ser Val Pro
                155                 160                 165
Glu Leu Val Pro Val Lys Val Ala Asn His Thr Gly Cys Lys Cys Leu
            170                 175                 180
Pro Thr Ala Pro Arg His Pro Tyr Ser Ile Ile Arg Arg Ser Ile Gln
        185                 190                 195
Ile Pro Glu Glu Asp Arg Cys Ser His Ser Lys Lys Leu Cys Pro Ile
    200                 205                 210
Asp Met Leu Trp Asp Ser Asn Lys Cys Lys Cys Val Leu Gln Glu Glu
215                 220                 225                 230
Asn Pro Leu Ala Gly Thr Glu Asp His Ser His Leu Gln Glu Pro Ala
                235                 240                 245
Leu Cys Gly Pro His Met Met Phe Asp Glu Asp Arg Cys Glu Cys Val
            250                 255                 260
Cys Lys Thr Pro Cys Pro Lys Asp Leu Ile Gln His Pro Lys Asn Cys
        265                 270                 275
Ser Cys Phe Glu Cys Lys Glu Ser Leu Glu Thr Cys Cys Gln Lys His
    280                 285                 290
Lys Leu Phe His Pro Asp Thr Cys Ser Cys Glu Asp Arg Cys Pro Phe
295                 300                 305                 310
His Thr Arg Pro Cys Ala Ser Gly Lys Thr Ala Cys Ala Lys His Cys
                315                 320                 325
Arg Phe Pro Lys Glu Lys Arg Ala Ala Gln Gly Pro His Ser Arg Lys
            330                 335                 340
Asn Pro
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(A) NAME/KEY: Other
(B) LOCATION: 13
(C) OTHER INFORMATION: Xaa = any amino acid

(A) NAME/KEY: Other
(B) LOCATION: 15
(D) OTHER INFORMATION: Xaa is any amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Cys Xaa Cys Xaa Cys
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC10917

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GCCACACATG ATGTTTGACC AAG
23

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC10924

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CTTCAACATC ATGTGGTGTG GCAG
24

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC10920

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6.

AGGATGTGTG GTGACAGCTG CA
22

(B) INFORMATION FOR SEQ ID NO:7

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC10919

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7.

CCTGCAGCTG TCAGGACAGA TG
22

(C) INFORMATION FOR SEQ ID NO:8.

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC10919

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8.

(TTTTTTT TTTCCAGCAGG TAT
33

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(v) IMMEDIATE SOURCE:
(B) CLONE: ZC10923

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CCCACAGCCG AAAGAATCCT TGAT
24

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC11782

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GGTGGTACCA TGTACAGAGA GTGGGTA
27

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: [illegible]
(B) TYPE: [illegible]
(C) STRANDEDNESS: [illegible]
(D) TOPOLOGY: [illegible]

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC11783

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

ACTGGATCCA GGATCTTTG GGCTGT
26

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vi) IMMEDIATE SOURCE:
(B) CLONE: ZC11826

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CGTGGTACCA TGTACAGAGA GTGCGTAGTG
30

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

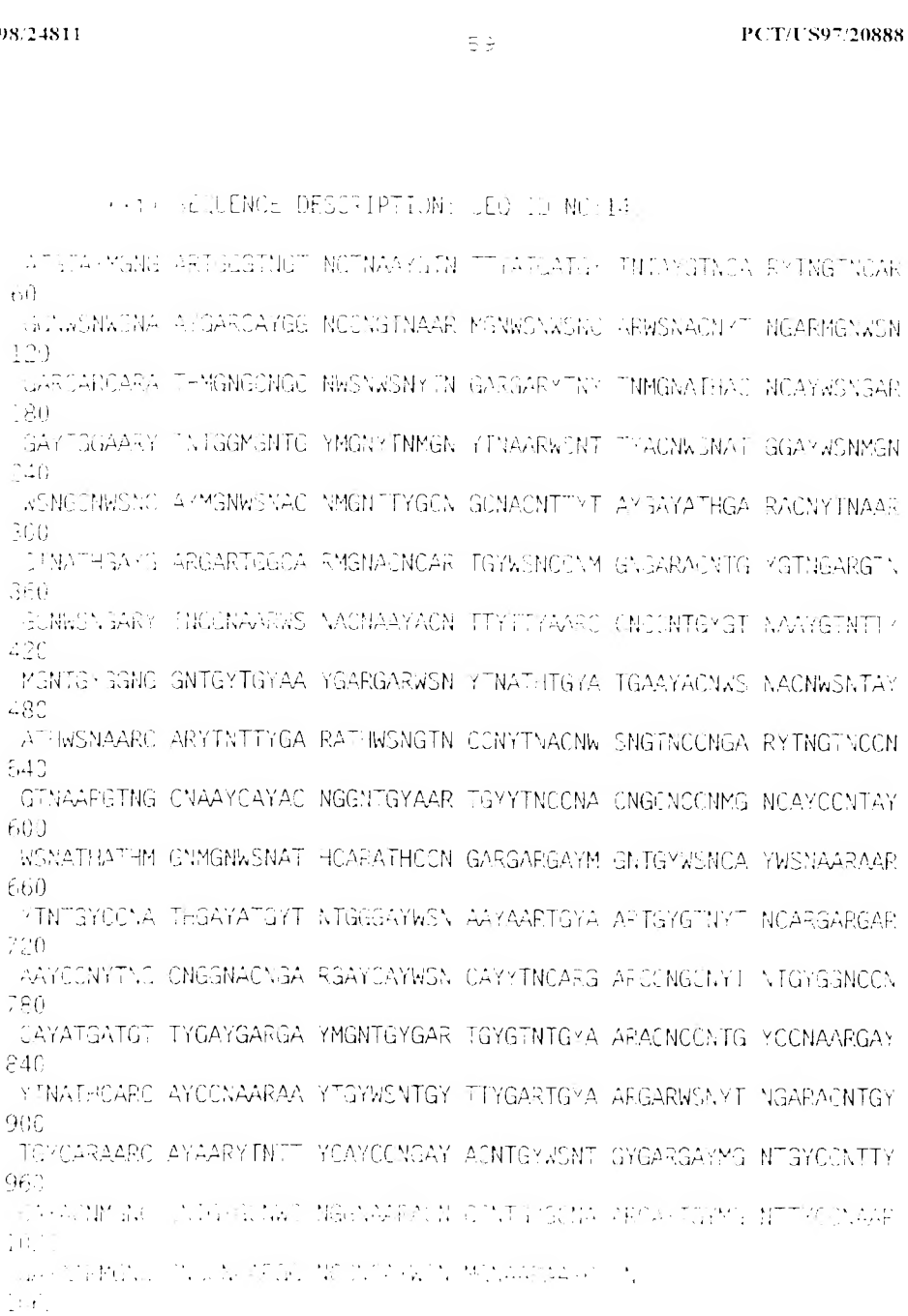
(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC11c27

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13

ASTGCATGCG CGGGGGCGTG TTGGCAA
27

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1362 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear



CLAIMS

What is claimed is:

1. An isolated polypeptide comprising a sequence of amino acid residues that is at least 80% identical in amino acid sequence to residues 109 to 197 of SEQ ID NO:2, wherein said polypeptide dimerizes to form a protein that is mitogenic for fibroblasts or smooth muscle cells.

2. An isolated polypeptide according to claim 1, wherein said polypeptide is at least 90% identical in amino acid sequence to residues 109 to 197 of SEQ ID NO:2.

3. An isolated polypeptide according to claim 1 further comprising a Balbiani ring motif carboxyl-terminal to said sequence of amino acid residues.

4. An isolated polypeptide according to claim 1 comprising a sequence of amino acid residues as shown in SEQ ID NO:2 selected from the group consisting of:

residues 109-205;
residues 85-205;
residues 22-205;
residues 1-205;
residues 109-354;
residues 85-354;
residues 22-354; and
residues 1-354.

5. An isolated polypeptide according to claim 1 further comprising an affinity tag.

6. An isolated polypeptide according to claim 5 wherein said affinity tag is polyhistidine, protein A, glutathione S transferase, substance P, or an immunoglobulin heavy chain constant region.

7. An isolated polypeptide according to claim 5 further comprising a proteolytic cleavage site between said sequence of amino acid residues and said affinity tag.

8. An isolated polypeptide having an amino acid sequence selected from the group consisting of:

residues 109-205 of SEQ ID NO:2;
residues 85-205 of SEQ ID NO:2;
residues 22-205 of SEQ ID NO:2;
residues 1-205 of SEQ ID NO:2;
residues 109-354 of SEQ ID NO:2;
residues 85-354 of SEQ ID NO:2;
residues 22-354 of SEQ ID NO:2; and
residues 1-354 of SEQ ID NO:2.

9. An isolated protein dimer having two polypeptide chains, wherein each of said chains comprises a sequence of amino acid residues that is at least 90% identical in amino acid sequence to residues 109 to 197 of SEQ ID NO:2, wherein said protein is mitogenic for fibroblasts or smooth muscle cells.

10. An isolated protein dimer according to claim 9 wherein each of said chains is at least 95% identical in amino acid sequence to residues 109 to 197 of SEQ ID NO:2.

11. An isolated protein dimer according to claim 9 wherein at least one of said chains further

comprises a Balbiani ring motif carboxyl-terminal to said sequence of amino acid residues.

12. An isolated protein dimer according to claim 9 wherein each of said polypeptide chains comprises a sequence of amino acid residues as shown in SEQ ID NO:2 individually selected from the group consisting of:

residues 109-205;
residues 85-205;
residues 22-205;
residues 1-205;
residues 109-354;
residues 85-354;
residues 22-354; and
residues 1-354.

13. An isolated protein dimer according to claim 9 wherein each of said polypeptide chains has an amino acid sequence individually selected from the group consisting of:

residues 109-205 of SEQ ID NO:2;
residues 85-205 of SEQ ID NO:2;
residues 22-205 of SEQ ID NO:2;
residues 1-205 of SEQ ID NO:2;
residues 109-354 of SEQ ID NO:2;
residues 85-354 of SEQ ID NO:2;
residues 22-354 of SEQ ID NO:2; and
residues 1-354 of SEQ ID NO:2.

14. A polypeptide produced by a method comprising:

culturing a cell containing a DNA construct comprising the following operably linked elements:

a transcription promoter;

a DNA segment encoding a polypeptide that is at least 90% identical to the amino acid sequence of SEQ ID NO:2 from residue 22 to residue 354; and

a transcription terminator; and

isolating the polypeptide encoded by said DNA segment and produced by said cell.

15. A polypeptide according to claim 14, wherein said DNA construct further comprises a secretory signal sequence operably linked to said DNA segment.

16. A polypeptide according to claim 14, wherein said DNA segment encodes a polypeptide that is at least 90% identical in amino acid sequence to residues 22 to 354 of SEQ ID NO:2.

17. A polypeptide according to claim 14, wherein said DNA segment encodes a polypeptide that is at least 95% identical in amino acid sequence to residues 22 to 354 of SEQ ID NO:2.

18. A dimeric protein produced by a method comprising:

culturing a cell containing a DNA construct comprising the following operably linked elements:

a transcription promoter;

a secretory signal sequence;

a DNA segment encoding a polypeptide that is at least 90% identical to the amino acid sequence of SEQ ID NO:2 from residue 22 to residue 354; and

a transcription terminator,

whereby said DNA segment is expressed and said polypeptide is dimerized to form a dimeric protein; and

isolating the dimeric protein from said cell.

19. A protein according to claim 18, wherein said DNA segment encodes a polypeptide that is at least 90% identical in amino acid sequence to residues 22 to 304 of SEQ ID NO:2.

20. A protein according to claim 18, wherein said DNA segment encodes a polypeptide that is at least 95% identical in amino acid sequence to residues 22 to 304 of SEQ ID NO:2.

21. An isolated polynucleotide encoding a polypeptide according to any of claims 1-8.

22. An isolated polynucleotide according to claim 21 wherein said polynucleotide is DNA.

23. An isolated polynucleotide according to claim 21 which is from 999 base pairs to 2500 base pairs in length.

24. An expression vector comprising the following operably linked elements:

a transcription promoter;

a DNA segment encoding a zvegf2 polypeptide according to any of claims 1-8; and

a transcription terminator.

25. An expression vector according to claim 24 further comprising a secretory signal sequence operably linked to said DNA segment.

26. A cultured cell into which has been introduced an expression vector according to claim 24, wherein said cell expresses the DNA segment and produces a polypeptide encoded by the DNA segment.

27. A cultured eukaryotic cell into which has been introduced an expression vector according to claim 25, wherein said cell expresses the DNA segment and secretes a polypeptide encoded by the DNA segment in the form of a dimeric protein.

28. A method of producing a dimeric protein comprising:

culturing a eukaryotic cell into which has been introduced an expression vector according to claim 25, whereby said said polypeptide is secreted from the cell as a dimeric protein that is mitogenic for fibroblasts or smooth muscle cells; and

recovering said dimeric protein.

29. An antibody that specifically binds to a polypeptide according to any of claims 1-8.

30. An antibody that specifically binds to a dimeric protein having two polypeptide chains, wherein each of said chains comprises a sequence of amino acid residues that is at least 80% identical in amino acid sequence to residues 109 to 197 of SEQ ID NO:2, wherein said protein is mitogenic for fibroblasts or smooth muscle cells.

31. A method of promoting cell growth, comprising incubating eukaryotic cells in a culture medium comprising a dimeric protein having two polypeptide chains, wherein each of said chains comprises a sequence of amino acid residues that is at least 80% identical in amino acid sequence to residues 109 to 197 of SEQ ID NO:2, in an amount sufficient to stimulate mitogenesis in said cells.

32. A method according to claim 31 wherein said cells are fibroblasts or smooth muscle cells.

33. A method of identifying an inhibitor of cell mitogenesis, comprising:

providing cells responsive to a dimeric protein having two polypeptide chains, wherein each of said chains comprises a sequence of amino acid residues that is at least 80% identical in amino acid sequence residues 109 to 197 of SEQ ID NO:2, wherein said protein is mitogenic for fibroblasts or smooth muscle cells;

culturing a first portion of said cells in the presence of said dimeric protein;

culturing a second portion of said cells in the presence of said dimeric protein and a test sample; and

detecting a decrease in a cellular response of said second portion of said cells as compared to said first portion of said cells.

34. A method of detecting a growth factor antagonist, comprising assaying a test sample for the ability to reduce binding of a protein to a receptor, wherein said protein is a dimeric protein having two polypeptide chains, wherein each of said chains comprises a sequence of amino acid residues that is at least 80% identical in amino acid sequence residues 109 to 197 of SEQ ID NO:2, wherein said protein is mitogenic for fibroblasts or smooth muscle cells.

Fig. 1

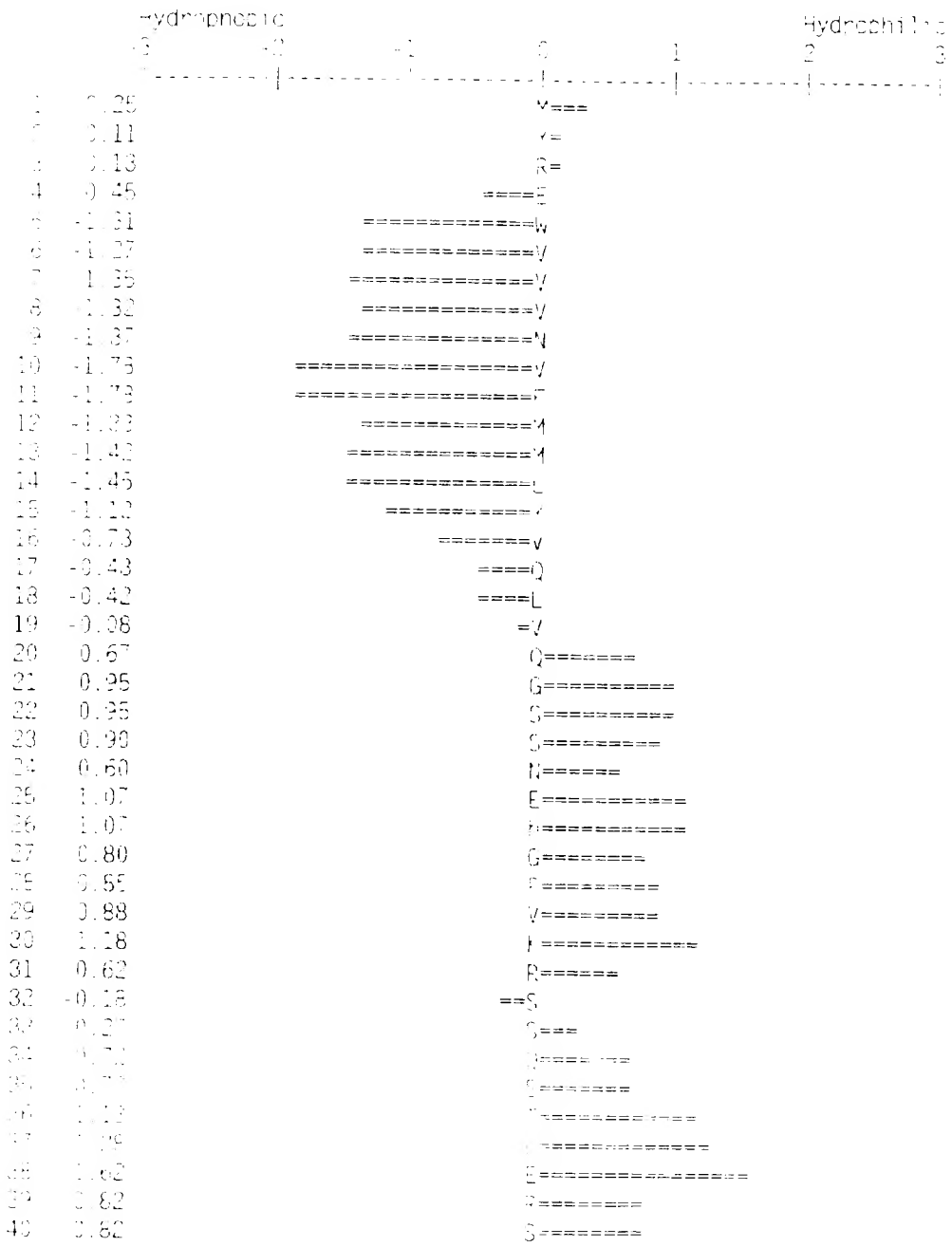

Hydrophobic
Hydrophilic
-3
-2
-1
0
1
2
3
1 0.25
2 0.11
3 0.13
4 0.45
5 -1.81
6 -1.27
7 1.36
8 -1.32
9 -1.87
10 -1.76
11 -1.78
12 -1.33
13 -1.42
14 -1.45
15 -1.13
16 -0.73
17 -0.43
18 -0.42
19 -0.38
20 0.67
21 0.95
22 0.95
23 0.90
24 0.60
25 1.07
26 1.07
27 0.80
28 0.55
29 0.88
30 1.18
31 0.62
32 -0.18
33 0.41
34 0.13
35 0.79
36 1.19
37 1.08
38 1.02
39 0.82
40 0.62

Fig. 1

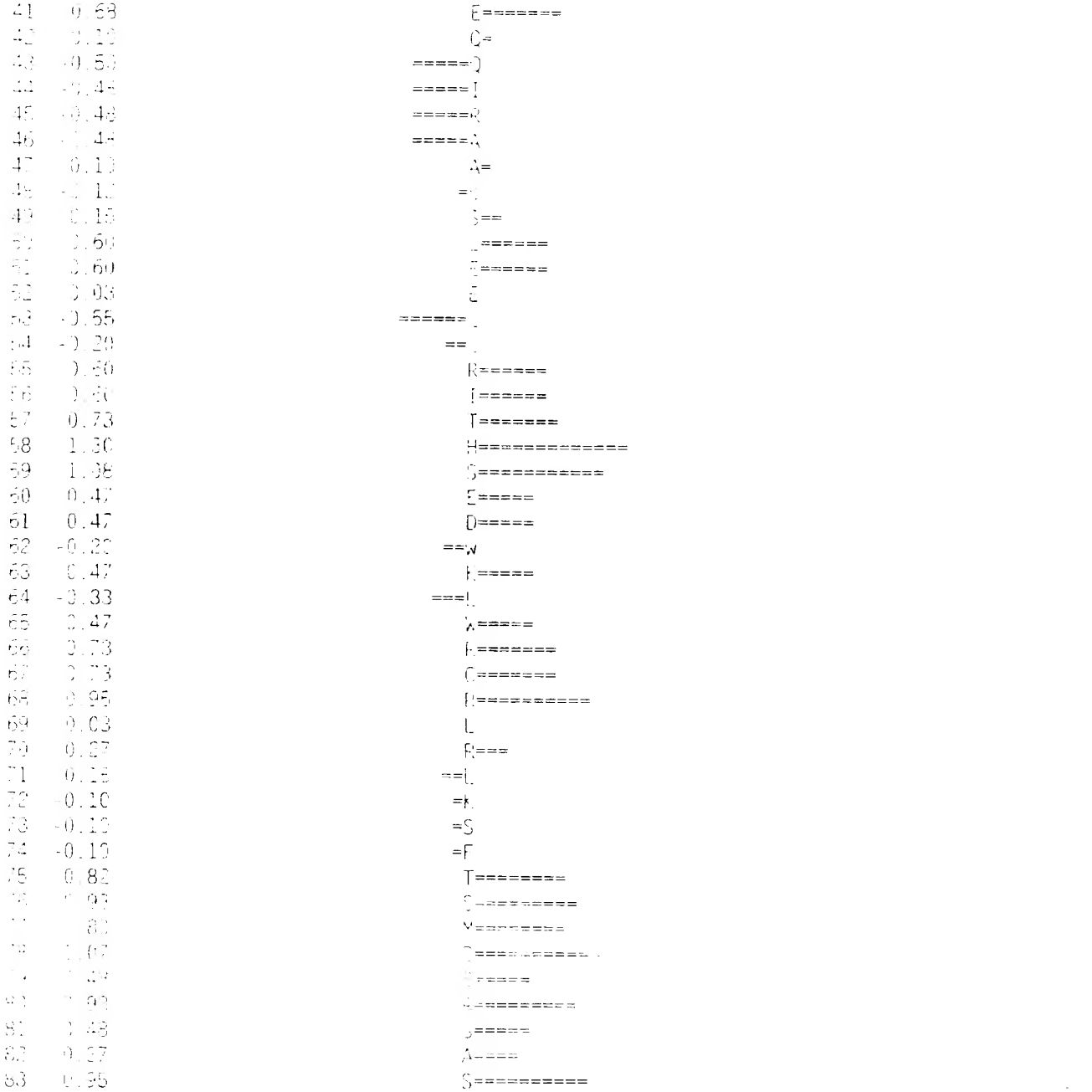

41 0.68
43 -0.50
44 -0.44
45 -0.48
47 0.13
49 0.15
50 0.60
51 0.60
52 0.03
53 -0.55
54 -0.20
55 0.60
57 0.73
58 1.30
59 1.08
60 0.47
61 0.47
62 -0.22
63 0.47
64 -0.33
65 0.47
66 0.73
67 0.73
68 0.95
69 0.03
70 0.27
71 0.15
72 -0.10
73 -0.10
74 -0.10
75 0.82
81 0.48
82 0.27
83 0.95

Fig. 1

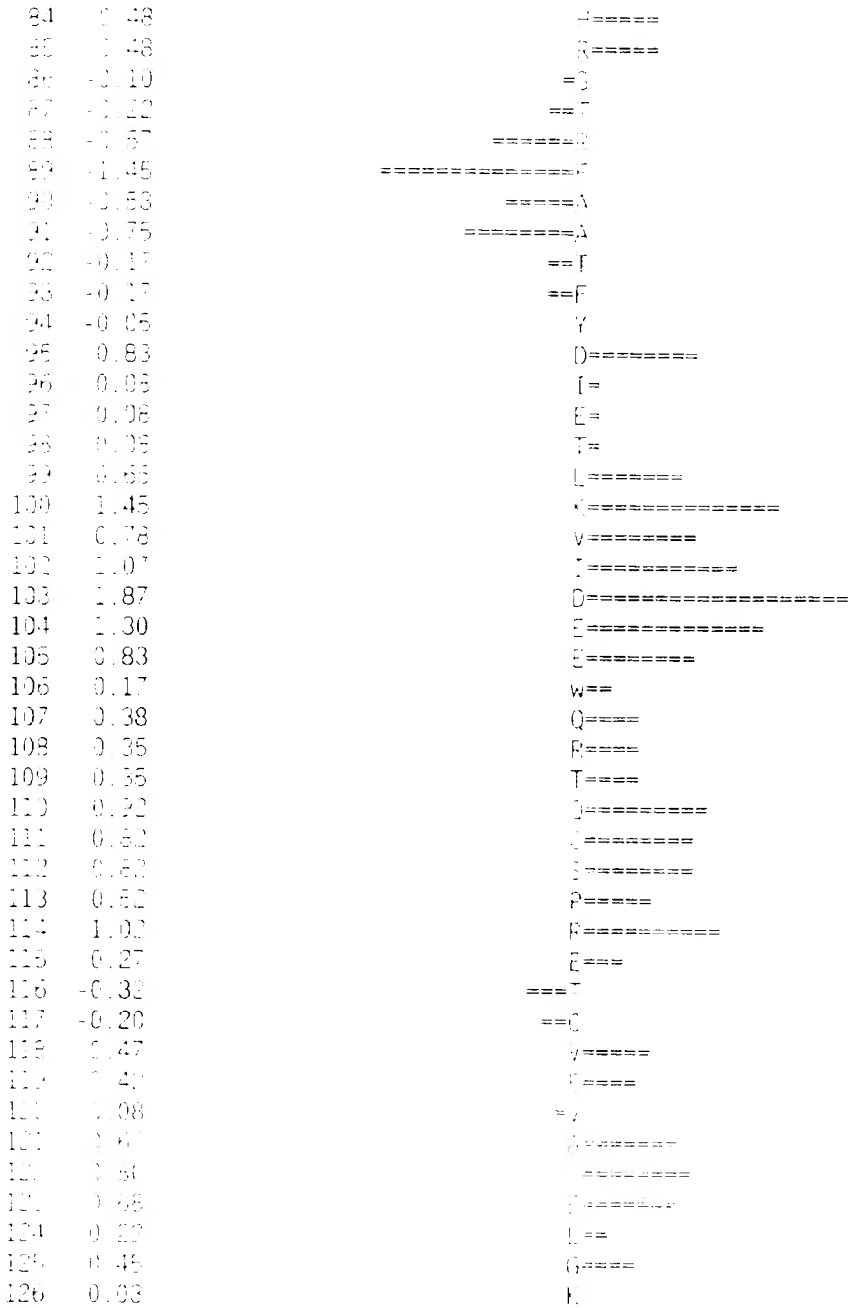

84 0.48
85 1.48
86 -0.10
87 -1.22
88 -1.97
89 -1.45
90 -0.53
91 -0.75
92 -0.17
93 -0.17
94 -0.05
95 0.83
96 0.08
97 0.08
98 0.09
99 0.65
100 1.45
101 0.78
102 1.07
103 1.87
104 1.30
105 0.83
106 0.17
107 0.38
108 0.35
109 0.55
110 0.92
111 0.62
112 0.62
113 0.52
114 1.02
115 0.27
116 -0.32
117 -0.20
118 0.47
119 0.49
120 1.08
121 0.67
122 0.84
123 0.48
124 -0.20
125 0.45
126 0.03

Fig. 1

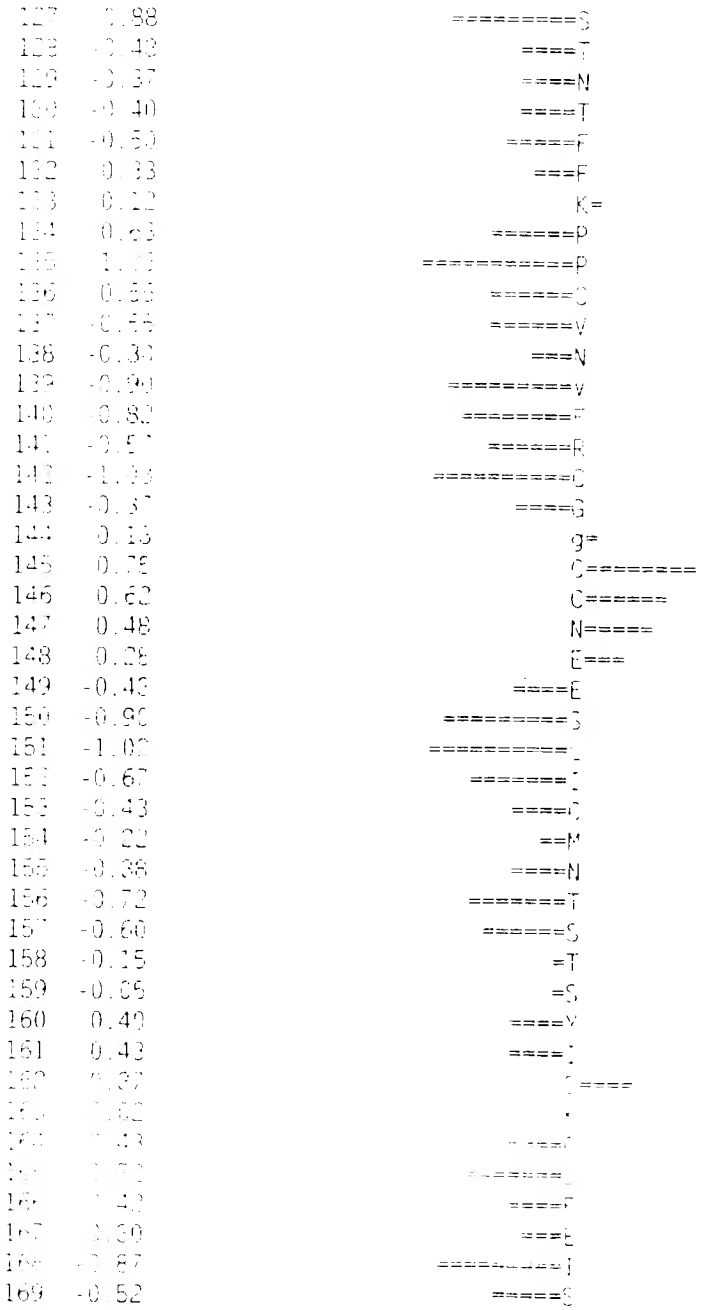

127 -1.88 =========S
128 -2.40 ====T
129 -2.37 ====N
130 -2.40 ====T
131 -2.50 =====F
132 -2.33 ===F
133 -2.22 K=
134 -2.03 ======P
135 -2.33 ==========P
136 -2.33 ======C
137 -2.33 ======V
138 -2.30 ===N
139 -2.90 =========V
140 -2.82 ========F
141 -2.57 ======R
142 -2.33 ==========C
143 -2.37 ====G
144 -2.13 g=
145 -2.22 C========
146 -2.62 C======
147 -2.48 N=====
148 -2.28 E===
149 -2.43 ====E
150 -2.90 =========S
151 -3.02 ==========
152 -2.67 =======
153 -2.43 ====C
154 -2.22 ==M
155 -2.38 ====N
156 -2.72 =======T
157 -2.60 ======S
158 -2.15 =T
159 -2.05 =S
160 -2.42 ====V
161 -2.43 ====I
162 -2.37 I====
163 -2.62 .
164 -2.33 -===
165 -2.22 -======
166 -2.43 ====F
167 -2.30 ===E
168 -2.67 ==========I
169 -2.52 =====S

Fig. 1

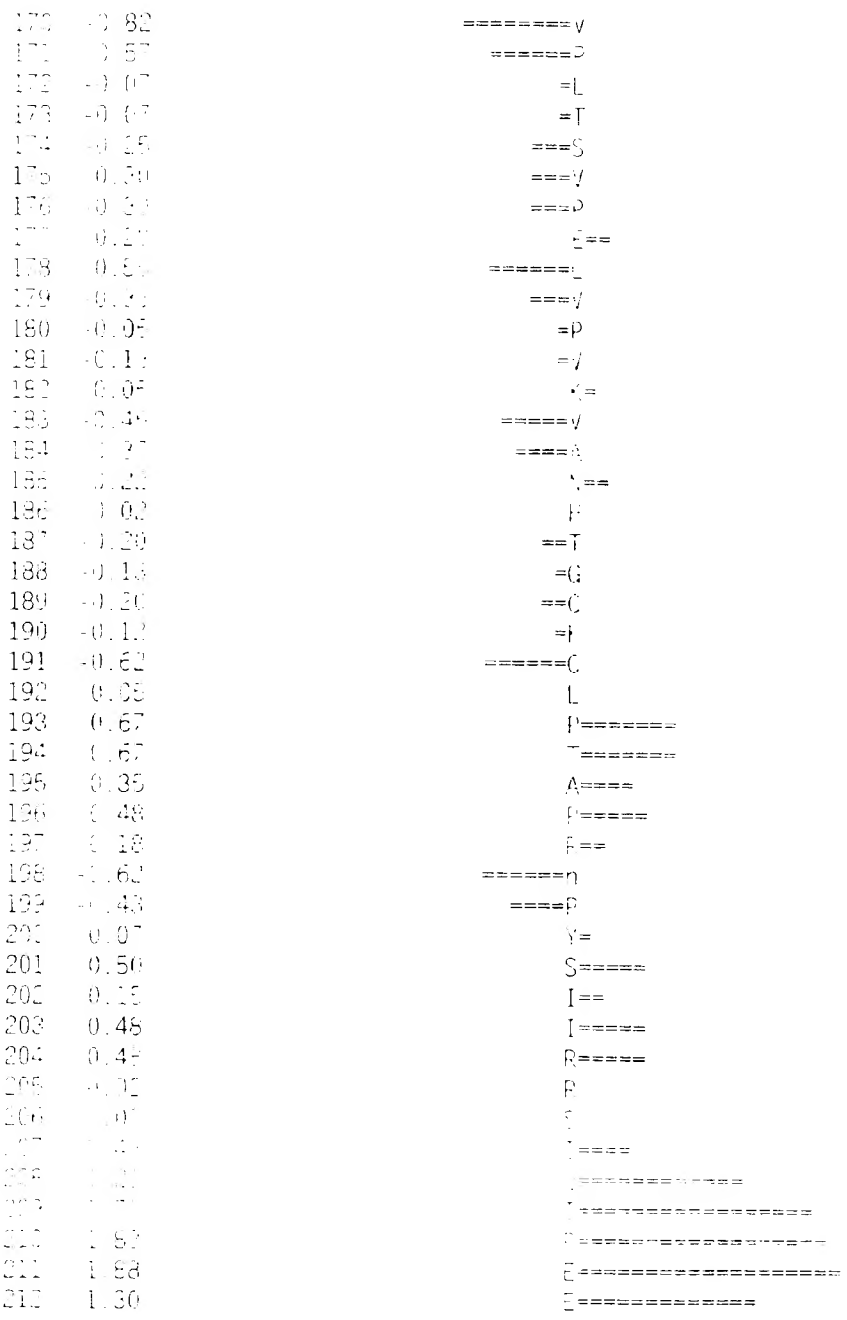


Fig. 1

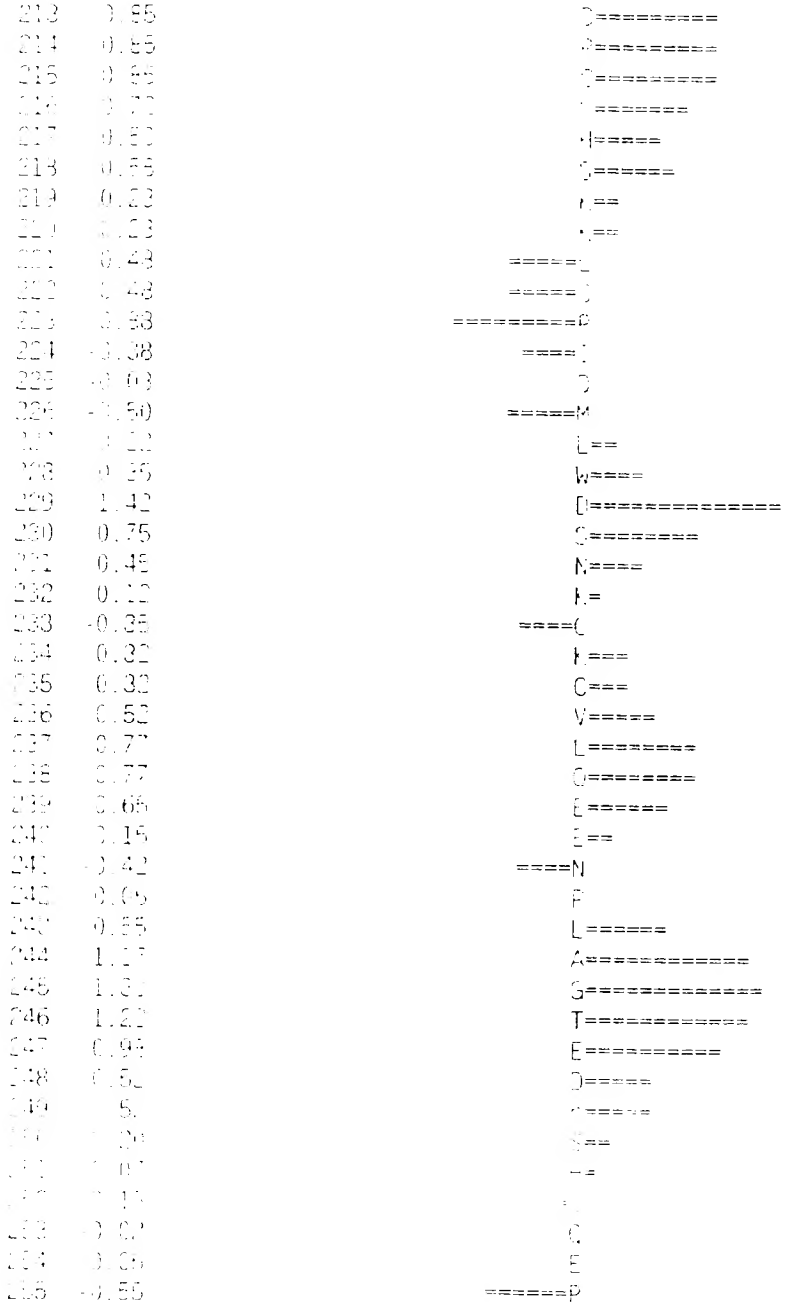


Fig. 1

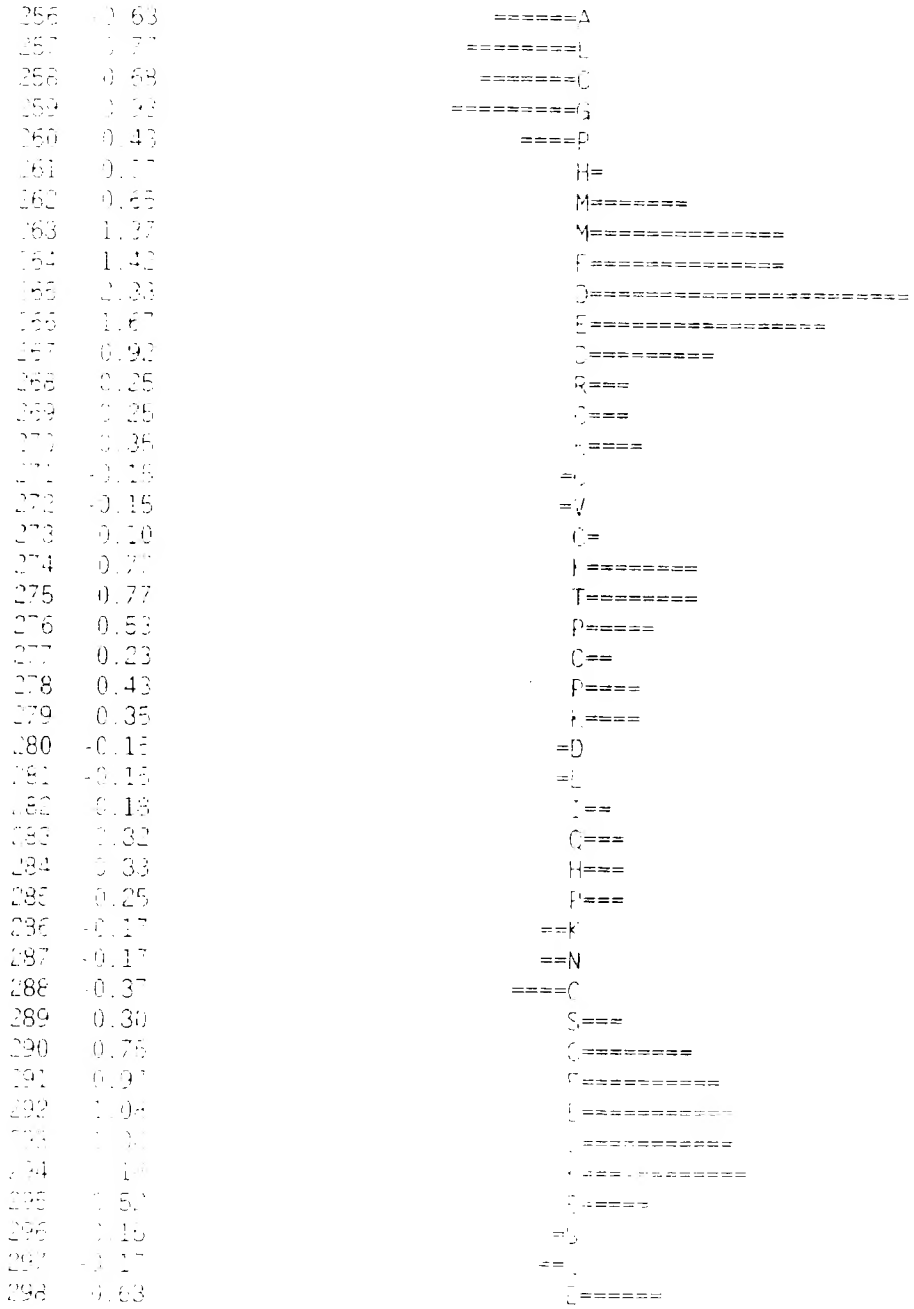

256 -0.63
258 -0.68
259 0.23
260 0.43
261 0.17
262 0.65
263 1.27
264 1.42
265 2.33
266 1.67
267 0.92
268 0.25
269 0.25
270 0.35
271 -0.18
272 -0.15
273 0.10
274 0.27
275 0.77
276 0.53
277 0.23
278 0.43
279 0.35
280 -0.13
281 -0.13
282 0.18
283 0.32
284 0.33
285 0.25
286 -0.17
287 -0.17
288 -0.37
289 0.30
290 0.75
291 0.97
292 1.03
294 1.47
295 1.53
296 1.15
297 0.17
298 -0.63
======A
========L
=======C
=========G
====P
H=
M=======
M==========
F==========
D=====================
E==================
C=========
R===
S===
T====
=Y
=V
Q=
I========
T========
P=====
Q==
P====
K====
=D
=L
L==
Q===
H===
P===
==K
==N
====C
S===
S========
C==========
L==========
A==========
V==========
S=====
=B
==Z
E=======

Fig. 1

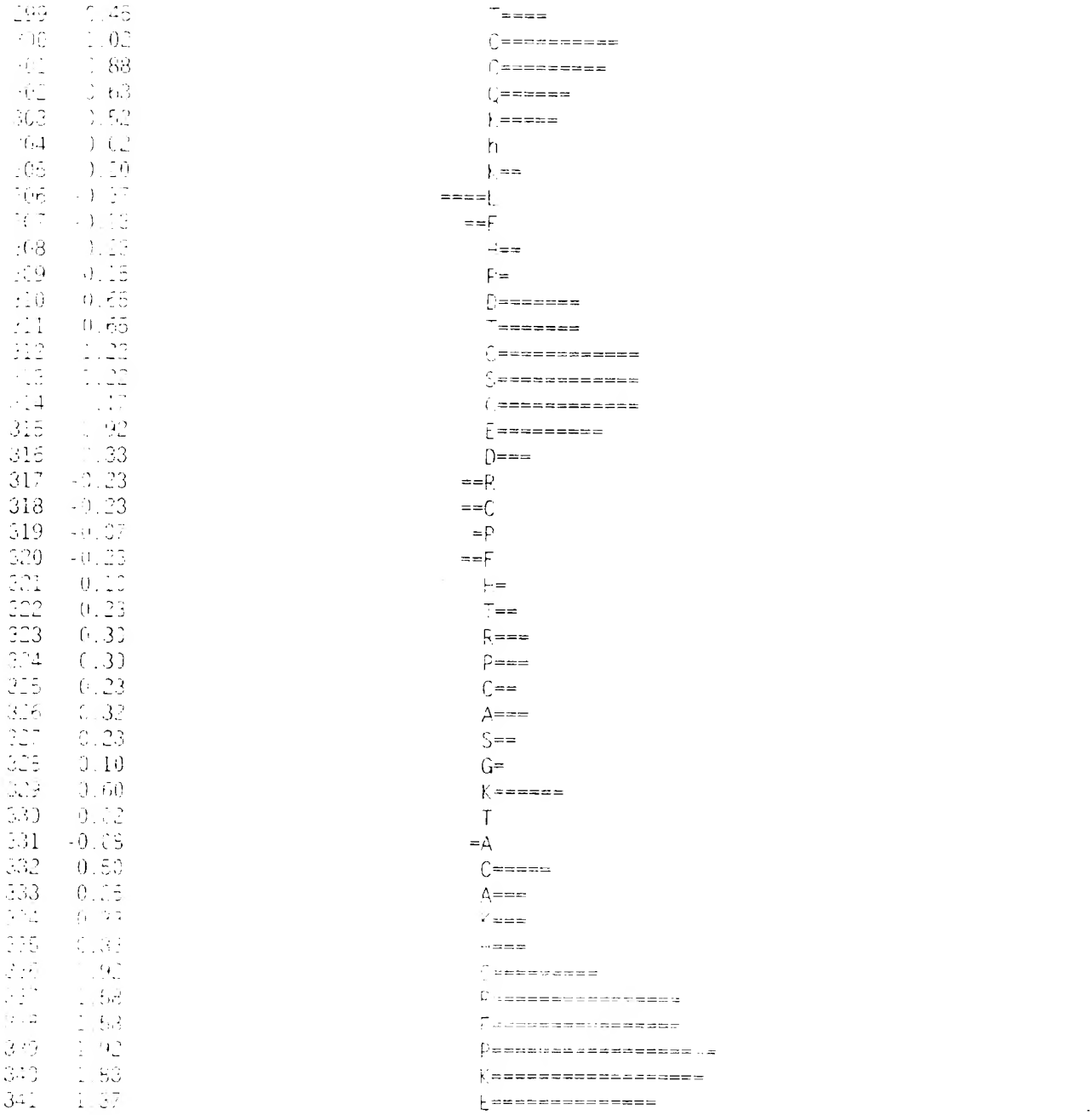

299 0.46
300 1.02
301 1.88
302 2.63
303 2.52
304 2.02
305 2.10
306 1.97
307 1.13
308 0.23
309 -0.15
310 0.56
311 0.65
312 1.33
313 1.33
314 1.17
315 1.92
316 1.33
317 -0.23
318 -0.23
319 -0.07
320 -0.23
321 0.10
322 0.23
323 0.30
324 0.30
325 0.23
326 0.32
327 0.23
328 0.10
329 0.60
330 0.02
331 -0.05
332 0.50
333 0.05
334 0.93
335 0.93
336 1.90
337 1.64
338 1.64
339 1.92
340 1.93
341 1.37

Fig. 1

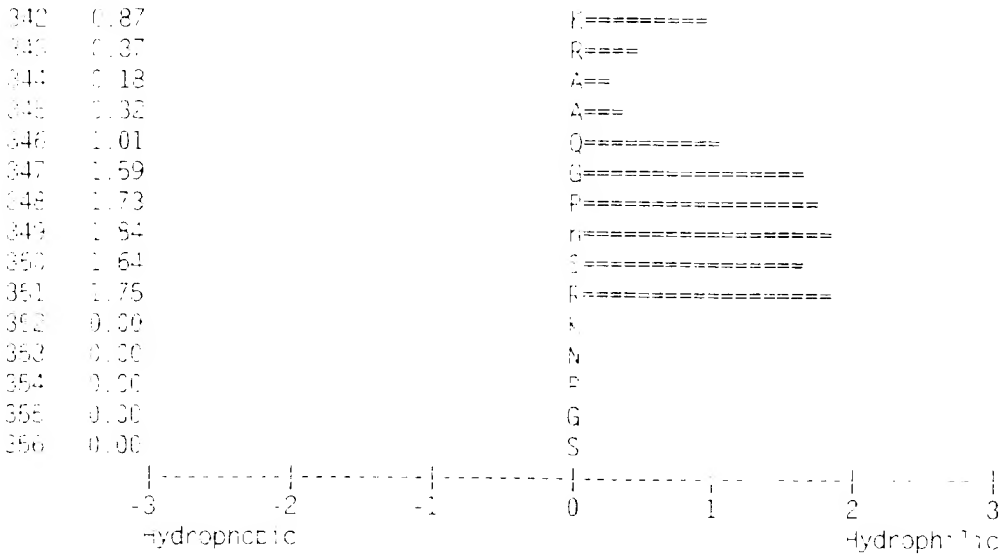
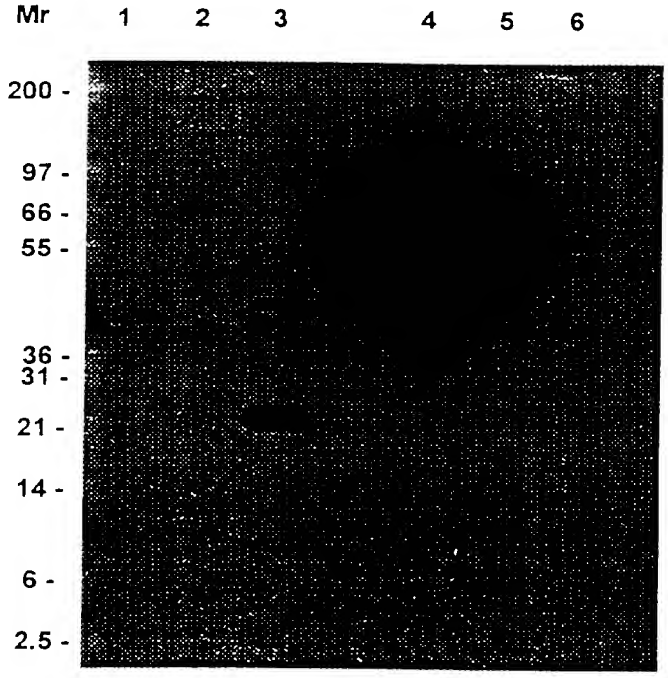

342 0.87
343 0.37
344 0.18
345 1.32
346 1.01
347 1.59
348 1.73
349 1.84
350 1.64
351 1.75
352 0.00
353 0.00
354 0.00
355 0.00
356 0.00
-3
-2
-1
0
1
2
3
Hydrophobic
Hydrophilic

Fig. 2



INTERNATIONAL SEARCH REPORT

Int... tional Application No
PCT/US 97/20888

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/18 C12N15/79 C12N5/06 C07K14/475 C07K16/18
C12P21/02 A61K38/18 G01N33/53

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	YAMADA, Y. ET AL.: "Molecular cloning of a novel Vascular Endothelial Growth Factor, VEGF-D" GENOMICS, vol. 42, no. 3, 15 June 1997, pages 483-488, XP002073018 see page 484 - page 485; figure 1 *'Materials and Methods' and 'Results'* --- -/--	1-4,8, 14-17, 21-26

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search
29 July 1998

Date of mailing of the international search report
13/08/1998

Name and mailing address of the ISA
European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+31-70) 340-3016

Authorized officer

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/20888

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication where appropriate, of the relevant passages	Relevant to claim No
P,X	WO 97 12972 A (UNIVERSITA 'DEGLI STUDI DI SIENA) 10 April 1997 see page 3, line 13 - line 18 see page 7, line 4 - page 9, line 26 see page 10, line 7 - line 22 see page 11, line 31 - line 37 see page 17, line 29 - page 19, line 16 see page 22, line 3 - page 27, line 11; figures 1,2	1-3,5,6, 9-11, 14-27, 29-32

X	ORLANDINI, M. ET AL.: "Identification of a c-fos-induced gene that is related to the platelet-derived growth factor/vascular endothelial growth factor family" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 93, October 1996, WASHINGTON US, pages 11675-11680, XP002029340	1-3,5,6, 9-11,14, 15,18, 21-27, 29-32
Y	see the whole document	7,28

Y	NILSSON, B. ET AL.: "Expression and purification of recombinant insulin-like growth factors from Escherichia coli" METHODS IN ENZYMOLOGY, vol. 198, 1991, pages 3-16, XP002073019 cited in the application see page 5 - page 7 *Fusions to Staphylococcal Protein A* see figures 1-3	7

Y	WO 96 26736 A (LUDWIG INSTITUTE FOR CANCER RESEARCH) 6 September 1996	28
A	see page 8, line 1 - line 5 see page 15, line 33 - line 36; example 16	1-27, 29-32

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int ...tional Application No
PCT/US 97/20888

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9712972	A	10-04-1997	AU	7142996 A	28-04-1997
			EP	0853668 A	22-07-1998
WO 9626736	A	06-09-1996	US	5607918 A	04-03-1997
			AU	5302196 A	18-09-1996
			CA	2211687 A	06-09-1996
			EP	0814827 A	07-01-1998
			NO	973933 A	01-09-1997